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OPHTHALMIC DRUG DEVELOPMENT: LOG P_{OW}, LIPOPHILICITY AND ION-PAIRING BY HPLC

Master of Science Thesis

Examiner: Prof. Timo Laaksonen
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ABSTRACT

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Due to the ageing population, the rate of the eye diseases has increased globally. Thus, there is a great need to develop more effective ophthalmic drugs and dosing methods. Ocular diseases are often treated with eye-drops or ointments. However, typically less than 5 % of the drug reaches the target tissue, since major part of the dose is systemically absorbed via nasolacrimal system. The main barrier for ocular absorption is the tightness of corneal epithelium, the outer most layer of the eye. In addition, the sensitivity of the eye presents challenges for the development of the optimal ophthalmic formulations.

Drug discovery and development is an economically risky and long lasting process. It takes an average of 10-15 years before a new drug is available to the patients. Even today, drug development relies heavily on animal testing so there is pressure to develop ethically more acceptable methods to replace or support animal testing. Especially in the early stages of drug development *in silico* computational and computer-based methods are widely used for mapping new potential drug molecules. In addition, simple physicochemical properties such as lipophilicity, ionization and solubility can be measured to predict how the drug is absorbed, distributed and excreted from the body. Absorption processes can also be modelled by various *in vitro* cell models and artificial lipid membranes. These methods can be used to select the most potential drug candidates for further animal studies and clinical trials on humans.

In this thesis, it was studied if the Quality Control and Analytical Development Laboratory of Santen Oy could utilize HPLC instruments and simple shake-flask method in modelling the factors affecting lipophilicity of the ophthalmic drugs and hence predict their absorption potential. Lipophilicity of two commercial isopropyl ester prodrugs and their biologically active metabolites were studied by C18-HPLC over a wide pH range to obtain pH-lipophilicity profiles. Moreover, it was studied if some specific interactions between these drug molecules and a certain drug additive could be revealed. The ion-pair formation between a common anti-glaucoma drug timolol and a sorbate additive was verified as increased lipophilicity by this method. In addition, a modified shake-flask method was developed for determining the octanol-water partition coefficients of drugs.

TIIVISTELMÄ

METSBERG, HANNA-KAISA: Silmälääkkeiden kehittäminen: Log P_{ow} ja lipofiilisyyden ja ioniparin muodostumisen määritys HPLC:llä
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Väestön ikääntymisen johdosta silmäsairaudet ovat yleistyneet, joten tarve kehittää tehokkaampia silmälääkkeitä ja toimivampia annostelutapoja on suuri. Silmäsairauksien hoidossa käytetään useimmiten silmän pinnalle annosteltavia silmätippoja tai -voiteita, joista imeytyy silmään tyypillisesti alle 5 %. Iso osa lääkkeestä kulkeutuu annostelun jälkeen kyynelkanavia pitkin nenänielun limakalvoille ja imeytyy systeemiseen verenkiertoon. Lääkeaineen imeytymistä rajoittaa erityisesti silmän pinnan sarveiskalvon tiivis epiteelisolukko. Lisäksi silmän herkkyys asettaa omat haasteensa optimaalisten lääkeformulaatioiden kehitykselle.

Lääketutkimus- ja kehitystyö on hidas ja taloudellisesti riskialtis prosessi. Uuden lääkkeen kehitys ja pääsy potilaskäyttöön vie keskimäärin jopa noin 10-15 vuotta. Vielä tänäkin päivänä tutkimuksissa käytetään runsaasti eläinkokeita, joten paine kehittää eläinkokeita korvaavia tai täydentäviä eettisesti hyväksyttävämpiä tutkimusmenetelmiä on suuri. Erityisesti lääkekehityksen alkuvaiheessa käytetään nykyään laajalti erilaisia laskennallisia ja tietokonepohjaisia menetelmiä potentiaalisten lääkeainemolekyylien kartoittamiseksi. Lisäksi yksinkertaisten fysikaaliskemiallisten ominaisuuksien, kuten dissosioitumisasteen sekä rasva- ja vesiliukoisuuden kartoittamisen avulla voidaan tehdä oletuksia siitä, miten lääke imeytyy, jakautuu ja erittyy elimistössä. Imeytymistä voidaan mallintaa myös erilaisten *in vitro* solumallien ja keinotekoisten lipidikalvojen avulla. Nämä menetelmät auttavat seulomaan potentiaalisimmat lääkekandidaatit jatkossa tehtäviin eläin- ja ihmiskokeisiin.

Tässä työssä tutkittiin, miten Santen Oy:n laadunvalvonta- ja analytiikan kehityslaboratoriossa voitaisiin HPLC-laitteistojen ja ravistelukokeiden avulla mallintaa lääkeaineiden rasvaliukoisuuteen vaikuttavia tekijöitä ja siten ennustaa lääkeaineen kykyä imeytyä silmään. Lääkeformuloinnissa käytettävän pH:n ja erään apuaineen vaikutusta kahden kaupallisen aihiosilmälääkkeen ja näiden biologisesti aktiivisten hajoamistuotteiden rasvaliukoisuuteen tutkittiin C18-HPLC laitteiston avulla. Menetelmän avulla onnistuttiin myös havainnollistamaan, kuinka sorbaattiapuaine lisää glaukooman hoidossa yleisesti käytetyn timololin rasvaliukoisuutta ioniparіutumisen vuoksi. Lisäksi työssä kehitettiin yksinkertainen ravistelukoemenetelmä, jolla voidaan määrittää lääkeaineiden rasvaliukoisuutta oktanoli-vesi-jaukautumiskertoimien avulla.

PREFACE

The research of this thesis was performed in the Quality Control and Analytical Development Laboratory of Santen Oy from September 2010 to January 2011. The impulse for this study arose from my interest in pharmaceutical chemistry after attending pharmacology and drug design courses at the University of Tampere.

I am very thankful to Veli-Matti Riipinen for providing me the idea of the thesis and the opportunity to work at Santen Oy. Your passion for reducing animal testing inspired me during the project. I would also like to thank Professor Robert Franzen for accepting the topic of the thesis and for supervising my work in cooperation with Veli-Matti. I also appreciate the help of my co-workers at Santen Oy. Special thanks go to Helvi for the guidance into the world of HPLC-analytics and to Antti for the excellent concentration measurements by mass spectrometry. I also thank Päivi Waris for proofreading and accepting my written results for the publication.

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Tampere, 29.7.2018

Hanna-Kaisa Metsberg

CONTENTS

ABBREVIATIONS AND SYMBOLS

AcOH	Acetate buffer
AGB	Alpha-1-acid glycoprotein
BAK	Benzalkonium chloride
BBB	Blood-brain barrier
BGE	Background electrolyte
BMC	Biopartitioning micellar chromatography
CHI	Chromatographic hydrophobicity index
CZE	Capillary zone electrophoresis
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EOF	Electro osmotic flow
GC	Gas chromatography
HCE	Human corneal epithelium
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IAM	Immobilized artificial membrane
ILC	Immobilized liposome chromatography
IPC	Ion-pair chromatography
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
log D	Logarithm of distribution coefficient D at a selected pH
log k	Logarithm of retention factor
log k_w	Logarithm of retention factor extrapolated to pure water
log P	Logarithm of partition coefficient P of neutral species
log P_{ow}	Logarithm of octanol-water partition coefficient
MeCN	Acetonitrile
MW	Molecular weight
NMR	Nuclear magnetic resonance spectrometry
NP	Normal phase
OECD	The Organisation for Economic Co-operation and Development
PACE	Pressure-assistant capillary electrophoresis
PAMPA	Parallel artificial membrane permeability assay
${}^{w}_{w}pH$	pH of the mobile phase before addition of organic solvent
${}^{s}_{w}pH$	pH of the mobile phase after addition of organic solvent
pK_a	Ionization constant
p$_o K_a$	Apparent ionization constant
PSA	Polar surface area
RP	Reversed phase

SGA	Spectral gradient analysis
SPB	Sodium phosphate buffer
TER	Transepithelial electrical resistance
THH	Timolol hemihydrate
UV	Ultra violet
$[A^-]$	concentration of deprotonated acid form
$[B]$	concentration of base
$[BH^+]$	concentration of conjugate acid
Δc	concentration gradient
c_o	concentration in organic phase
c_w	concentration in water phase
D	diffusion coefficient
h	effective thickness of diffusion barrier
$[HA]$	concentration of acid
J	flux across membrane per area unit per time unit
φ	volume fraction of organic solvent in mobile phase
φ_0	chromatographic hydrophobicity index
K	partition coefficient of the drug into the membrane
k	retention (capacity) factor
L_{eff}	effective capillary length
L_{tot}	total capillary length
μ_{eff}	effective mobility
P	permeability coefficient, partition coefficient
r	volume ration $V_{n\text{-}octanol}/V_{water}$
S	slope of the line
t_a	migration time of the analyte
t_{EOF}	migration time of the neutral marker
t_r	retention time
t_o	retention time of unretained component, void volume time
U	voltage
V_o	volume of organic phase
V_w	volume of water phase
X	drug additive
1a	acid metabolite of isopropyl ester prodrug **1e**
1e	commercial isopropyl ester prodrug
2a	acid metabolite of isopropyl ester prodrug **2e**
2e	commercial isopropyl ester prodrug

1. INTRODUCTION

Drug discovery and development is expensive and long lasting process which takes an average of 10-15 years (Rang & Hill 2012). Usually it includes drug discovery, product development, pre-clinical research and clinical trials on humans (Gad 2005). Speeding up drug discovery and development processes requires more effective and reliable research tools (Kraljevic et al. 2004). Even today, drug development relies heavily on animal testing so there is pressure to develop and apply ethically more acceptable methods (Badyal & Desai 2014). Especially in the early stages of drug development *in silico* computational and computer-based methods can be used for mapping new potential drug molecules (Ekins et al. 2007). In addition, simple physicochemical properties such as lipophilicity, ionization and solubility can be measured to predict how the drug is absorbed, distributed and excreted from the body (Henchoz et al. 2009). Absorption processes can also be modelled by various *in vitro* cell models and artificial lipid membranes (Agarwal & Rupenthal 2016; Kansy et al. 1998). These methods can be used to select the most potential drug candidates for further animal studies and clinical trials on humans. (Vellonen 2010)

Due to the ageing population, the rate of the eye diseases has increased globally (Flaxman et al. 2017). Thus, there is a great need to develop more effective ophthalmic drugs and dosing methods. Ocular diseases are often treated with eye-drops or ointments. However the target tissues for most ophthalmic drugs are located in the inner eye and typically less than 5 % of the drug reaches the site of action. The main barrier for ocular absorption is the tightness of corneal epithelium, but also systemic absorption from the conjunctiva and rapid elimination process by tear turnover limit the bioavailability of the ophthalmic drugs. Formulation researchers have traditionally tried to enhance ocular absorption either by increasing the permeability of the drug or by prolonging its corneal contact time. (Järvinen et al. 1995; Furrer et al. 2008) Contact time can be prolonged for example by increasing the viscosity of the drugs or by solid drug-releasing inserts, which unfortunately give patients a sensation of a foreign body in the eye (Furrer et al. 2008). In addition, drug permeability can be enhanced by chancing pH or osmolality of the drug solution, by using penetration enhancers and high permeability prodrugs or by encapsulating drugs into nanoparticles and liposomes (Furrer et al. 2008; Xu et al. 2013). The sensitivity of the eye presents challenges for the development of the optimal ophthalmic formulations, because if the formulation causes discomfort, enhanced lacrimation increases the drug loss (Furrer et al. 2008).

Literature review of this thesis is aimed at employees with non-pharmaceutical education background starting their work in ophthalmic drug development. The thesis presents the factors that affect the absorption of drugs, especially ophthalmic drugs. In addition, various models for drug permeation studies are introduced. The emphasis is on the determination of physicochemical properties such as lipophilicity and ionization constant. Lastly, liquid chromatography methods for pharmaceutical permeation studies are summarized.

Experimental part of the thesis was performed in the Quality Control and Analytical Development Laboratory of Santen Oy. The aim of the study was to develop in-house physicochemical laboratory models to support ophthalmic formulation development at Santen Oy. The main idea for experimental study was to find out if common RP-HPLC instruments and simple shake-flask method could be utilized in modelling the lipophilicity of the ophthalmic drugs. The first goal was to find out how the effect of the formulation pH could be studied by HPLC. Another aim was to test whether the HPLC measurements or shake-flask method could reveal drug-additive interaction.

Experimental part is divided into three different chapters. In the first chapter, lipophilicity of two commercial isopropyl ester prodrugs and their biologically active metabolites are studied by HPLC. For each compound, lipophilicity expressed as log k, is measured over a wide pH range to obtain pH-lipophilicity profiles which could be utilized while choosing the optimal formulation pH. Moreover, it is studied if some specific interactions, such as ion pairing between these drug molecules and a certain drug additive could be revealed with a C18-HPLC-system by adding increasing amount of the additive into the eluent. In the second chapter, a modified shake-flask method is set up for determining direct log P_{ow} values of drugs at Santen Oy. Lipophilicity (log P_{ow}) of an isopropyl ester prodrug with and without additive molecules is directly measured by this method in order to compare results obtained in previous chapter by RP-HPLC method. In the third chapter, the C18-HPLC method for studying drug-additive interactions is validated by using common anti-glaucoma drug timolol and a sorbate additive, for which ion pairing had been previously verified by NMR (Higashiyama et al. 2007). Before ion pairing studies, the eluent composition was optimized for timolol. Additionally, the pH-lipophilicity profile of timolol is measured.

2. DRUG ABSORPTION

Whatever the route of drug administration (e.g. parenteral, oral, percutaneous, pulmonary, ocular), drug must usually pass through at least one body membrane before reaching the target receptor. This movement is called absorption. Membranes are biological barriers that selectively inhibit the passage of drug molecules. Rate of the absorption depends both on the physicochemical properties of the drug and the membrane properties (Seydel & Wiese 2002).

2.1. Structure and lipid composition of cell membranes

Cell membranes are composed primarily of lipid bilayer. Membranes also contain embedded membrane proteins, cholesterol and oligosaccharide chains on the cell membrane surface (Figure 1). Although the lipid layer is highly organized it behaves as a dynamic fluid. According to the fluid mosaic model the biological membranes behave as two-dimensional liquid where phospholipids are able to move in the lateral plane of the bilayer. (Singer et al. 1972)

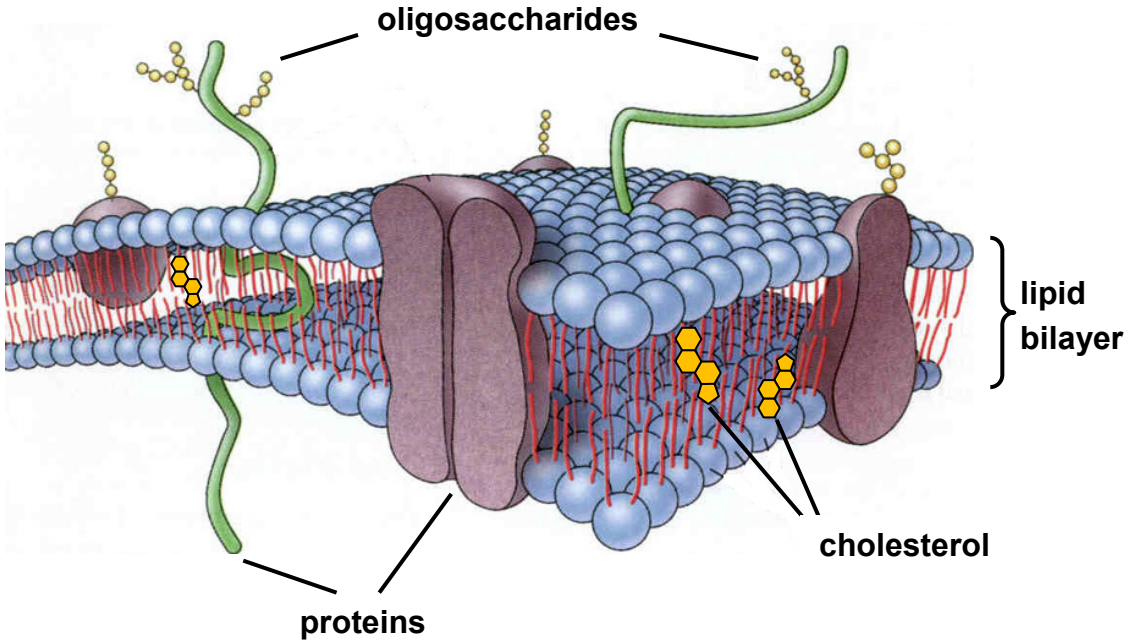

Figure 1. *Simplified schematic presentation of an animal cell membrane (modified from ref. Stern 1996).*

Lipid and protein composition of mammalian membranes varies in different species and organs. Therefore drug distribution and accumulation is cell organelle dependent. The common building blocks of lipid bilayer are however universal. There are three main classes of membrane lipids: phospholipids, glycolipids, and cholesterol. Phospholipids, the major component of cell membranes, can be further subdivided into glycerophospholipids and sphingosine-containing phospholipids. Most phospholipids have a glycerol backbone. These glycerophospholipids consist of two long-chain fatty acids which are esterified with hydroxyl groups of glycerol. The remaining hydroxyl group of glycerol is esterified to phosphoric acid which is further substituted by some sugar or amino alcohol such as choline, serine or ethanolamine. (Seydel & Wiese 2002; McGraw-Hill 1982)

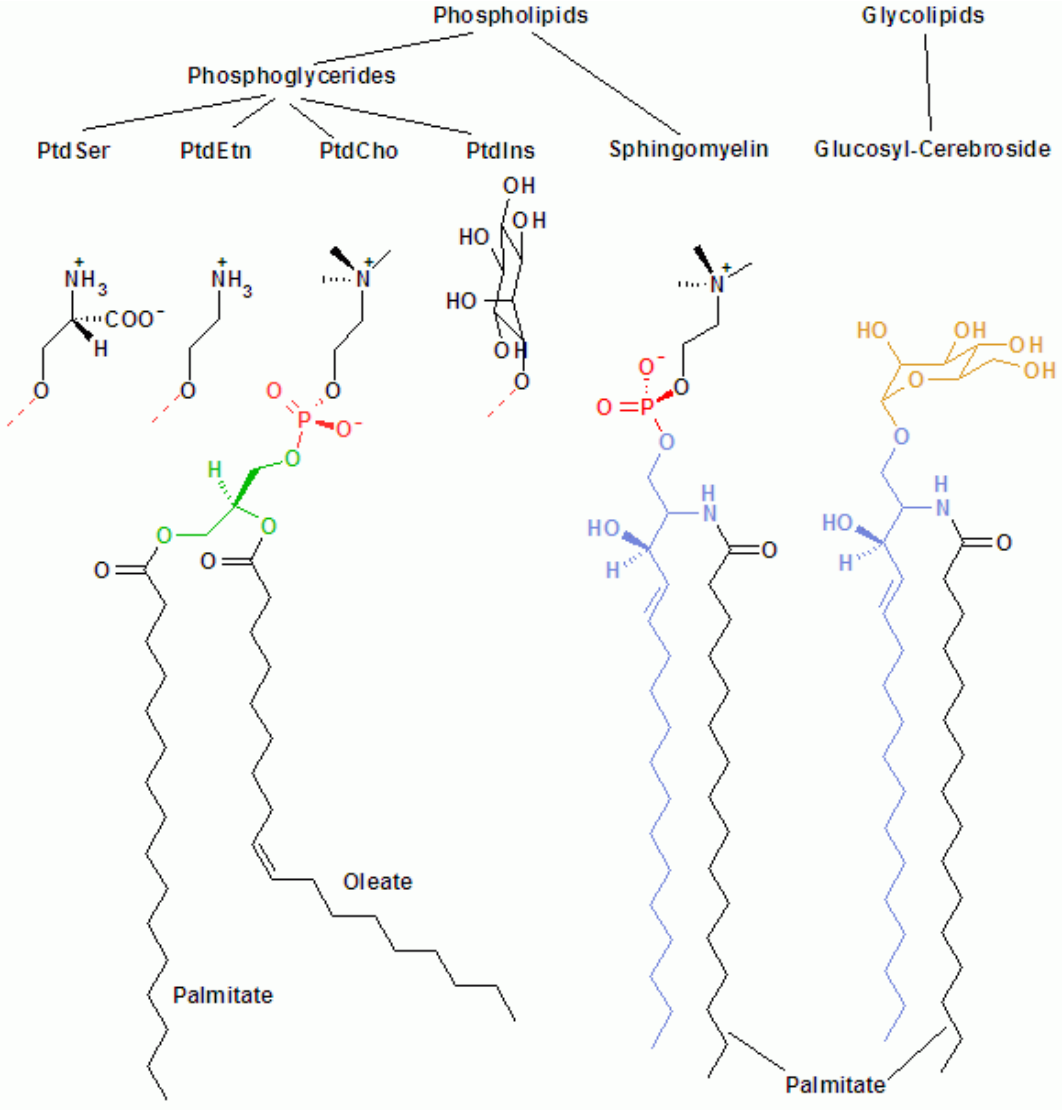

Figure 2. *Classification and the structural formulas of the most common amphipathic membrane lipids. (Wikipedia 2018)*

Sphingolipids are derived from sphingosine instead of glycerol. All sphingolipids have a ceramide backbone in which a fatty acid chain is attached to amino alcohol sphingosine by an amide linkage. Sphingomyelins are the only sphingolipid class that belongs also to phospholipids. Sphingomyelins have a phosphocholine or phosphoethanolamine molecule esterified to the 1-hydroxy group of a ceramide. (Seydel & Wiese 2002; McGraw-Hill 1982) Glycolipids contain one or more monosaccharide residues bound by a glycosidic linkage to a hydrophobic moiety such as an acylglycerol, a sphingoid, a ceramide or a prenyl phosphate (Chester 1997).

Most membrane lipids are amphipathic. The polar head groups and phosphate groups are hydrophilic, whereas non-polar fatty acid moieties are lipophilic. Thus, phospholipids are likely to spontaneous form bilayers in water. The hydrophobic fatty acids tails are facing each other and the hydrophilic polar head groups are exposed to the surrounding aqueous medium. Compared to the other membrane lipids, cholesterol is only weakly amphipathic since it has a single hydroxyl group (Figure 3). Therefore it is located in the hydrophobic region of the lipid bilayer. The increase of cholesterol restricts the mobility of membrane due to the rigid steroid ring structure. (Seydel & Wiese 2002)

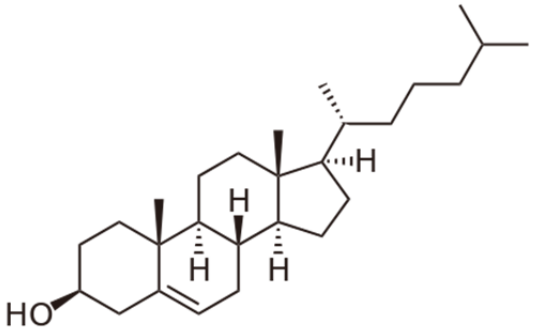

Figure 3. *The structural formula of cholesterol.*

2.2. Drug absorption across biological membranes

Drugs can be absorbed across any epithelial cell layer by two principal routes, transcellular or paracellular (Figure 4). In paracellular route drugs are transported between cells via water-filled intercellular junctions, whereas in the transcellular route drugs must pass first through the apical membrane, then across cytoplasm and finally through the basolateral membrane. Lipophilic drugs prefer the transcellular route because of the lipidic nature of cell membrane, while the paracellular route is preferred by hydrophilic drugs. (Bermejo 2008) The major barrier in the paracellular route is the tight junction located between the epithelial cells. The tightness, ion selectivity and number of these junctions vary among epithelia significantly. (Anderson 2001)

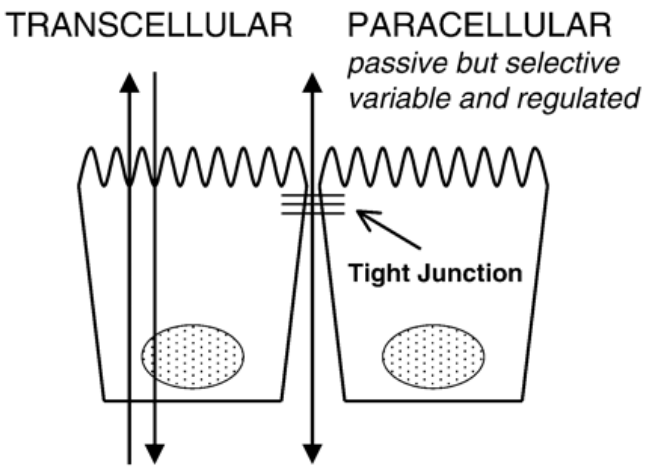

Figure 4. *Transepithelial transport pathways (Anderson 2001).*

Transcellular absorption can be divided into passive diffusion and carrier mediated transport mechanisms, such as facilitated diffusion and active transport (Figure 5). Facilitated diffusion is a movement of drug molecules down a concentration gradient via special transport proteins. It does not require external energy unlike active transport in which drugs can be transported against concentration gradient. Since these transport mechanisms are mediated by transporter proteins, the extent of facilitated diffusion and active transport is highly depended on the binding affinity between the drug and the protein. Carrier mediated transportation is therefore very selective and saturates easily since at high concentration levels all carrier proteins are occupied. (Bermejo 2008)

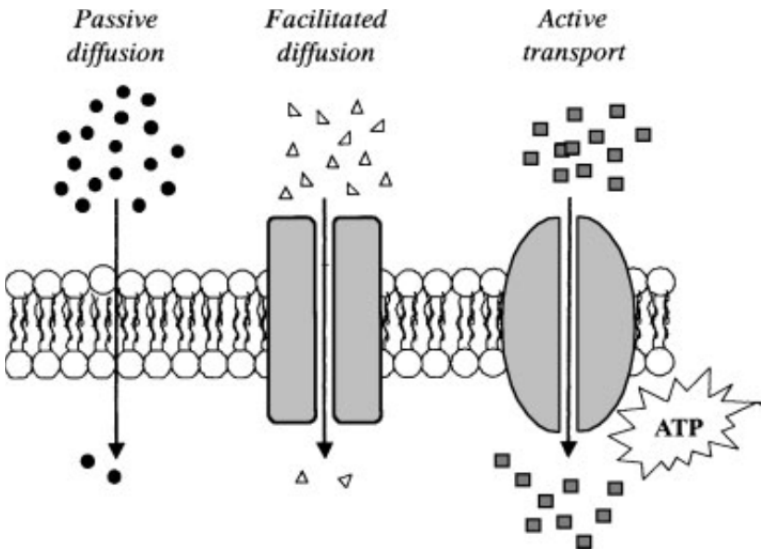

Figure 5. *Different transcellular absorption mechanisms across cell membrane. (van Balen et al. 2004)*

The predominant absorption route for drug compounds is passive transcellular diffusion. This absorption route requires that the compound can first leave behind the surrounding water molecules, pass the polar head groups of lipids, permeate through the lipophilic membrane interior, and then enter again to the hydrophilic surroundings on the other side of the membrane. Passive transcellular diffusion is driven by a concentration gradient from a region of high concentration to a region of relatively lower concentration. (Kerns 2001) Amount of drug diffused across a membrane in certain time depends on the permeability of the drug and the concentration gradient across the membrane. Maximal drug absorption is achieved when the drug has optimal permeability and maximum concentration at the site of absorption. Mathematically drug flux across the membrane can be expressed by Fick's first law. When assuming that the concentration gradient across membrane is time-independent and linear, Fick's law can be simplified into following equation:

$$J = P\Delta c \tag{1}$$

where J is the drug flux across membrane per area unit per time unit, P is permeability coefficient and the Δc is the concentration gradient. Permeability coefficient can be further described by equation:

$$P = \frac{DK}{h} \tag{2}$$

where D is the diffusion coefficient of the drug within the membrane, K is the partition coefficient of the drug into the membrane and the h is the effective thickness of diffusion barrier i.e. membrane. (Loftsson et al. 2006; Brodin 2010) Partition coefficient is generally denoted also by symbol P, but in this context symbol K is chosen to avoid confusion with permeability.

Although membrane is the main barrier for most drugs, stagnant water layer adjacent to the membrane surface can behave as a diffusion barrier for drugs that readily permeate the lipidic membrane. The significance of this aqueous barrier depends on the absorption route. For example at skin surface the aqueous layer is usually very thin and therefore relatively insignificant for the overall absorption process. However, mucin layer on the surface of the eye or intestinal epithelium can significantly affect the overall diffusion barrier. Thus drugs must be hydrophilic enough to diffuse through the membrane covering water layer and lipophilic enough to be able to partition from the aqueous surface into the membrane. (Loftsson et al. 2006)

In addition to water solubility and lipophilicity, there are several physicochemical properties of drugs, such as charge, the degree of ionization, molecular size and shape, hydrogen bonding capacity and polar surface are, which have an effect on permeation

through biological membranes. These properties are discussed more detailed in next chapter.

Yet another factor to consider is the protein binding affinity of drugs. Some drug molecules may have tendency to bind to plasma or tissue proteins. Bound drug is mostly pharmacologically inactive, but since such a binding is usually reversible, proteins can act as a reservoir of drugs. However, only free drug molecule is capable of diffusing through the membrane. (Schmidt et al. 2010)

3. PHYSICOCHEMICAL PROPERTIES DETERMINING MEMBRANE PERMEATION

3.1. Molecular size and weight

Drug absorption is highly dependent on molecular size which can be estimated by molecular weight. Especially in paracellular route diffusion rates are limited by molar mass. The larger the molecular mass, the slower the diffusion. (Bermejo 2008) For example, corneal epithelium allows paracellular permeation of only small molecules. Molecular size 500 g/mol has been estimated to be the upper limit. Conjunctiva is however leakier due to larger intercellular pore sizes and higher pore density. Paracellular space of conjunctiva allows permeation of molecules which molecular mass range between 5 000 and 10 000 g/mol. (Hämäläinen et al. 1997a) However, for larger molecules other absorption routes such as facilitated diffusion or active transportation by endocytosis are still possible. (Bermejo 2008)

3.2. Ionization and charge

Many drugs are either weak organic acids, bases or their salts. In aqueous solutions weak electrolytes exist as in ionized or in molecular (non-ionized) form. The degree of ionization of these drugs in solutions is extremely dependent on the ionization constant (pK_a) of the molecule and the pH of the environment. Exceptions to this generalization are steroids and quaternary ammonium compounds, which dissociate completely at physiological pH values and behave as strong electrolytes. (Gynther et al. 2003)

For weak acids the relationship between ionization constant pK_a and pH value can be presented by Henderson-Hasselbalch equation:

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \tag{3}$$

where $[A^-]$ represents the concentration of deprotonated form and $[HA]$ the concentration of protonated form of the acid. Equation 3 illustrates that when pH is equal to the pK_a of the acid, there are present equal amount of protonated and deprotonated acid molecules. Decrease in pH value increases the amount of non-ionized form of the acid. Thus the lower the pK_a value of the drug is the stronger the acid is. (Gynther et al. 2003)

The same Henderson-Hasselbalch relationship holds for weak bases as well:

$$pH = pK_a + \log\frac{[B]}{[BH^+]} \quad (4)$$

When pH of the solution decreases, also the amount of non-ionized form of the weak base decreases. (Gynther et al. 2003)

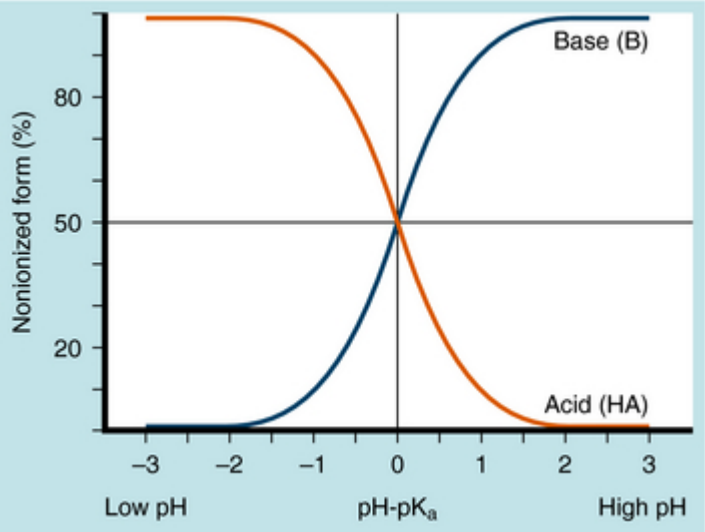

Figure 6. *Percentage of non-ionized drug as a function of pH for weak electrolytes having same pK_a values. When the pH is greater than the pK_a of an acidic drug the drug will be ionized. Conversely basic drugs will be ionized when the pH is lower than the drug's pK_a. (Crespo 2010)*

Situation is however more complicated when a drug has more than one acidic or basic center. Ampholytes for example can exist in acidic, basic, neutral or zwitterionic form depending on the pK_a values of both acidic and basic center. (Gynther et al. 2003)

According to the pH-partition theory, only the neutral form of the compound can permeate through the membrane. Conversely drugs are typically much more water soluble in their ionized form. (Gynther et al. 2003) Highly charged nature of cell membranes however complicates the situation. Most biological membranes contain lipids having acidic and zwitterionic head groups. Thus, the net charge of membrane surface is negative on both inner and outer surfaces of the cell. Negative surface potential attracts positive counterions from the surrounding solution to the interface which creates an electrical double layer adjacent to the membrane. Since surface potential increases the surface concentration of cations, they should permeate the membrane more readily than the anions. However on membrane-water interface lipid bilayer possess a membrane dipole potential that is positive in direction of the

membrane interior. This makes the interior of the bilayer more permeable to anions than cations, respectively. The origin of dipole potential is not entirely understood, but it has been assumed that it arises from the orientation of lipid head groups and the polarized water adjacent to the membrane surface. Additionally if there is a concentration difference of the ions between both sides of the membrane it will generate a transmembrane potential that carries ions along electrochemical gradient. (Mälkiä et al. 2004)

3.3. Lipophilicity and partition coefficient (log *P*)

As a measure of drug permeation in biological membranes, lipophilicity is one of the most important physicochemical parameters in predicting and interpreting membrane permeability. (Testa et al 1996) Lipophilicity represents the affinity of molecule or a moiety for a lipidic environment (van de Waterbeemd et al. 1997). Lipophilicity of a drug is usually expressed as log *P*, the logarithm of partition coefficient between two immiscible solvents, an organic and an aqueous phase:

$$\log P = \log \frac{c_o}{c_w} \tag{5}$$

where c_o and c_w represent the concentrations of the drug in organic and aqueous phase respectively. The greater the value of the partition coefficient is the more lipophilic the drug is. Partition coefficients are expressed as in logarithmic scale because of the broad range of variation (*P* = 0,0001-100 000). *N*-octanol and aqueous buffer solutions are most often used as solvents. (Gynther et al. 2003)

Partition coefficient is defined only for neutral (non-ionized) molecules. Many drugs are however weak electrolytes, as described earlier, and may be partially ionized depending of the pH. Since neutral and ionized molecules exhibit different polarities, their lipophilicities are also dissimilar. Thus the partition between organic and aqueous phase is pH dependent. When more than one electrical species are present in solution, log *P* is replaced by log *D* called distribution coefficient (also denoted as an apparent partition coefficient log P_{app}). Log *D* takes into account the intrinsic lipophilicity of electrical species present and their relative concentrations:

$$\log D = \log \frac{[neutral]_o + [ionized]_o}{[neutral]_w + [ionized]_w} \tag{6}$$

(Kah & Brown 2008)

If the pK_a value of the drug and the pH of the aqueous phase are known, partition coefficient can be calculated from distribution coefficient:

For monoprotic weak acids: $$\log P = \log D - \log\left[\frac{1}{1+10^{pH-pKa}}\right] \quad (7)$$

For monoprotic weak bases: $$\log P = \log D - \log\left[\frac{1}{1+10^{pKa-pH}}\right] \quad (8)$$

(Gynther et al. 2003)

On the other hand the effective lipophilicity log D of an acidic or basic compound at any pH value can be predicted if the dissociation constant pK_a and and partitition coefficient log P are known. For compounds having more than one ionizable group the relationship between log P and log D is obviously much more complex. (Smith et al. 2001)

Log P and log D values can be used to predict how likely drug candidates permeate cell membranes by transcellular passive diffusion. For drugs which permeate though the membranes by passive diffusion, the relationship between lipophilicity (log P or log D) and permeability is usually bell shaped. Drugs should not be too lipophililic but not too hydrophilic either. (Balimane et al. 2000)

3.4. Hydrogen bonding property and polar surface area

The surface of the cell membranes provides a finite amount of hydrogen bonding groups. Especially polar phospholipid head groups are excellent hydrogen bonding acceptors. Before partitioning into the non-polar interior of the lipid bilayer, a drug must break the hydrogen bonds with the surrounding water molecules and the polar head groups of membrane lipids. Breaking the bonds requires energy and therefore drugs must be lipophilic enough to overcome these energy losses. For drugs, which behave as strong hydrogen bonding donors, this can be rate limiting step in absorption process. Hence, high hydrogen bonding capacity has a negative effect on biological permeation. (Mälkiä et al. 2004)

The polar surface area (PSA) of a molecule characterizes the area of the polar part of the molecule. It is defined as a sum of the surface of all polar atoms, primarily oxygen and nitrogen, including also their attached hydrogens. PSA is a commonly used in medicinal chemistry to estimate drug's ability to permeate cell membranes, since it is easy to calculate and it takes into account those drug properties that has an important role in membrane permeation such as H-bonding features, molecular polarity and solubility. Molecules with a polar surface area of greater than 140 Å^2 permeate poorly cell membranes due to high polarity. (Mannhold et al. 2007)

3.5. Solubility

Solubility, or rather aqueous solubility is a major problem for drug formulation scientists, since nowadays many developed drugs are completely insoluble in water. Drug absorption process however requires drugs to be present in the form of solution at the site of absorption. Poorly soluble drugs can be modified by various methods to improve the solubility. For example in nanosuspensions particle size has been reduced to increase the surface area. Crystal engineering can be used to design crystallization conditions which affect the particle size, and thus, solubility as well. Solubility can be enhanced also by chemical methods such as ion-pair formation, complexation, the use of soluble prodrug or surfactants, or changing a pH by buffers. (Savjani et al. 2012)

In addition to the aqueous solubility measurements, nowadays it is more common to measure drug dissolution rates also in specific biological fluid compositions. Different kind of biorelevant media which can simulate the gastric, intestinal and colonic fluids has been developed and commercialized. (Bou-Chacra et al. 2017) Moreover simulated saliva, lung fluid, vaginal fluid, semen solutions, tears and sweat have been studied. (Marques et al. 2011)

4. OCULAR ABSORPTION

4.1. Anatomy of the eye

The main analytic elements of human eye are presented in Figure 7. Eye consists of an anterior and a posterior segment. Cornea, anterior chamber filled with aqueous humor, lens, iris and ciliary body belong to the anterior segment. The posterior segment consists of vitreous humor surrounded by retina, choroid, sclera and conjunctiva. Optic nerve conducts visual information from the retina to the brain. (Ahmed 2003)

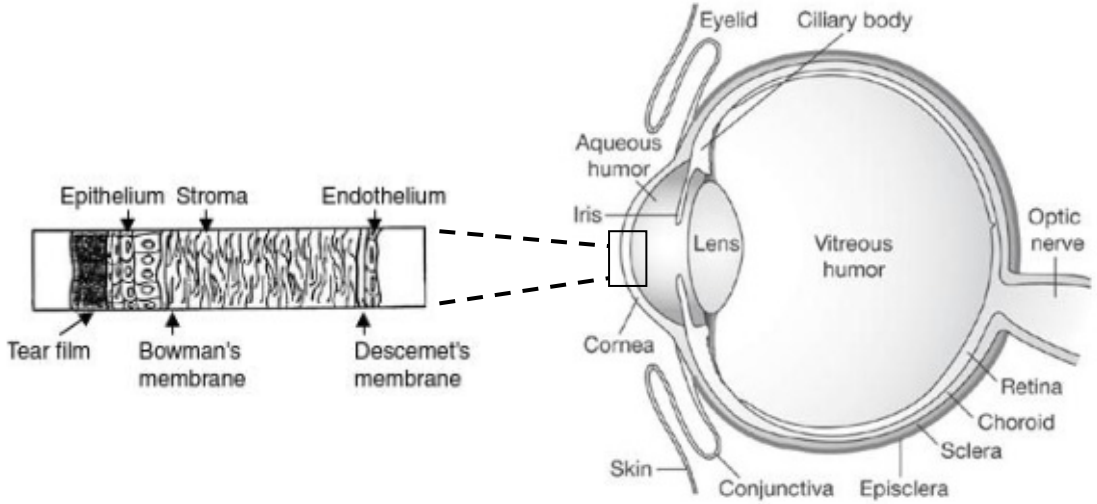

Figure 7. *The main analytic elements of human eye and schematic presentation of the cross section of cornea. Modified from ref. (Furrer et al. 2008; Smith et al. 2007).*

4.2. Absorption barriers

Ocular diseases are often treated with eye-drops or ointments. However the target tissues for most ophthalmic drugs are located in the inner eye and due to many biological barriers only a small fraction of the drug is absorbed in to the eye and reaches intraocular tissues. The main barrier for ocular absorption is the tightness of the corneal epithelium, but also lacrimal drainage and systemic absorption from the conjunctiva can wash away ocular drops. Basically drug absorption route from the surface of the eye can be either corneal or non-corneal. (Järvinen et al. 1995; Furrer et al. 2008) Due to the geometry and anatomy of the eyeball, drugs which are able to penetrate across cornea can reach the anterior segment of the eye such as cornea, aqueous humor and iris-ciliary body. In contrast, non-corneal absorption route across conjunctiva and sclera is more suitable for drugs which target tissues (choroid, retina, vitreous humor) locate in the posterior segment. (Ahmed 2003)

4.2.1. Corneal route of absorption

Cornea is a thin avascular tissue that is composed of three main layers: epithelium, stroma and endothelium. The cross section of the cornea is presented in Figure 7. In order to penetrate the cornea, drug should first partition into very lipophilic epithelium, from which it must partition into hydrophilic stroma and finally into relatively lipophilic endothelium. Thus, drugs must have some solubility in both lipidic and aqueous environment. Most ocular drugs are transported across cornea by passive diffusion. Molecular size and weight, the degree of ionization and the permeation coefficient of the drug affect the rate of passive diffusion. Hydrophobic epithelium represents a limiting barrier for highly hydrophilic compounds while the hydrophilic stroma limits the penetration of highly lipophilic compounds. The much leakier corneal endothelium allows both para- and transcellular permeation of various drugs. (Furrer et al. 2008)

Corneal epithelium is the main limiting barrier of drug absorption especially for hydrophilic drugs which must penetrate through paracellular route. Corneal epithelium has very tight intercellular junctions and epithelial pore size is estimated to be 2-3 nanometers. (Hämäläinen et al. 1997a and b; Lee et al. 1986) Therefore only hydrophilic and very small drugs can penetrate corneal epithelium paracellular. The isoelectric point of cornea is 3.2 and at physiological pH corneal epithelia is negatively charged which makes the paracellular space more permeable to positive ions than to negative ions. (Rojanasakul & Robinson 1989). Most ocular drugs are however lipophilic enough to permeate transcellularly across cornea. Optimal lipophilicity for corneal permeation has been reported to be log P = 2–3. (Schoenwald & Ward 1978; Schoenwald & Huang 1983)

4.2.2. Non-corneal route of absorption

Non-corneal drug absorption into the intraocular tissues includes penetration across both conjunctiva and sclera. Conjunctiva is a thin, transparent, moist and relative leaky mucous membrane that coats the inner surface of the eyelids and the white part of the eyeball called sclera. Conjunctiva has large surface area, rich blood flow and it secretes a thick mucus gel that lubricates the eye. (Watsky et al. 1988, Hämäläinen et al. 1997b; Ahmed 2003) Conjunctiva is more permeable to drugs than cornea since it has leakier epithelium. (Prausnitz & Noonan 1998; Hämäläinen et al. 1997a and b) Conjunctiva has also four times larger surface area compared to cornea (Ahmed 2003). The pore size of the conjunctival epithelium has been estimated to be two times larger and the pore density 16 times higher than in the corneal epithelium (Hämäläinen et al. 1997a). Therefore the size and the lipophilicity of the drug do not limit conjunctival absorption as significantly as corneal absorption (Toropainen 2007).

White sclera consists mainly of collagen and mucopolysaccharides (Keeley et al. 1984). It forms the supporting wall of the eyeball and is continuous with the transparent

cornea. Sclera maintains the shape of the eyeball by resisting intraocular pressure. It represents nearly 80 % of the surface area of the eyeball. (Battaglioli & Kamm 1984; Ahmed 2003) Sclera is generally more permeable tissue than conjunctiva and cornea (Toropainen 2007). Paracellular diffusion is assumed to be the main absorption mechanism across sclera. (Hämäläinen et al. 1997b)

Non-corneal route across conjunctiva and sclera plays significant role for hydrophilic drugs and compounds with large molecular size which are poorly absorbed across the cornea (Ahmed & Patton 1985; Ahmed et al. 1987; Chien et al. 1990; Hämäläinen et al. 1997a and b). The main problem of the non-corneal drug delivery is the risk of systemic absorption via conjunctival blood vessels and nasolacrimal system (Ahmed 2003).

4.3. Drug formulation development to enhance ocular absorption

Formulation development for ophthalmic drugs means designing a dosage form which provides a balance between absorption efficiency, formulation stability, sterility and ocular tolerance (see Figure 8). There are several ways to enhance ocular absorption and therapeutic efficacy of topically administered ophthalmic drugs. Generally these methods can be divided into two categories. Bioavailability can be enhanced either by increasing ocular penetration or contact time.

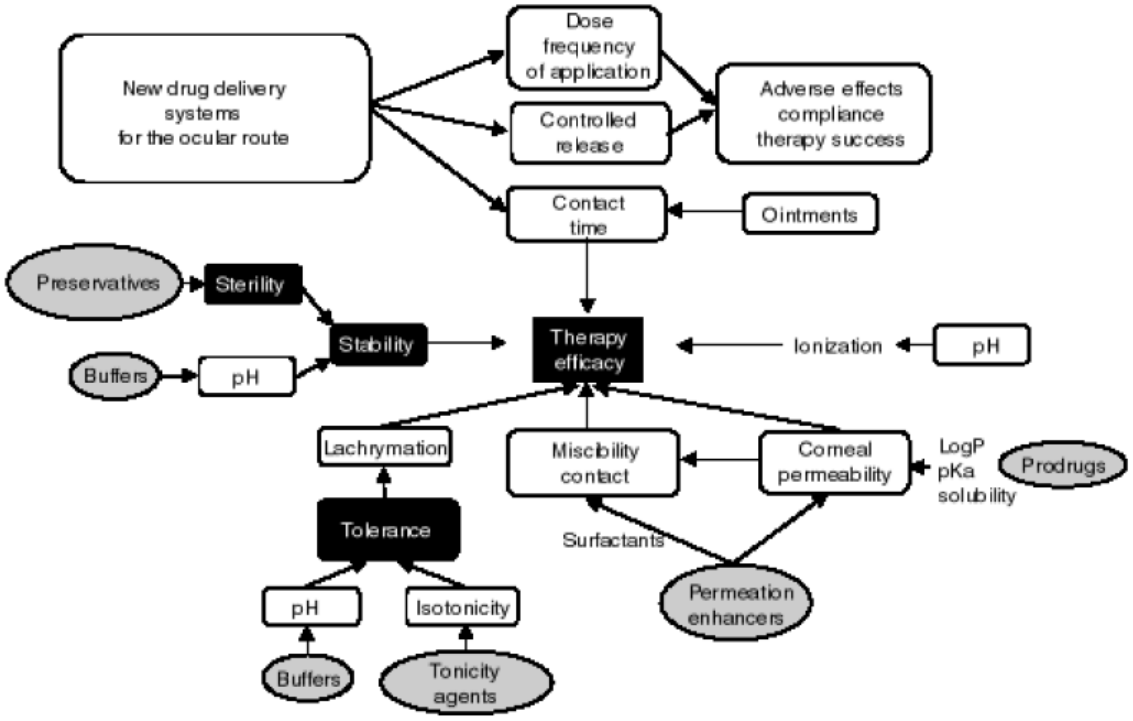

Figure 8. *Formulation parameters involved in topical ophthalmic drug development (Furrer et al. 2008).*

Contact time can be prolonged for example by increasing the viscosity of the drug solution (Ding 1998; Kaur & Kanwar 2002). Ophthalmic gels, suspensions and ointments are however difficult to apply and their dosing is more inaccurate compared to eye drop solutions. Increased viscosity causes blurred vision which restricts the usage at day time. (Bapatla & Hecht 1996) Sticky sensation also induces lacrimation and reflex blinking which leads to faster elimination of the drug from the surface of the eye (Furrer et al. 2008; Dudinski 1983).

New drug delivery systems are developed constantly. Contact time can be increased by using solid inserts which release drug at a constant rate for a certain time. These inserts can be either biodegradable, soluble or insoluble. The main problem of the inserts is that they give patients a sensation of a foreign body in the eye. Insoluble inserts also have to be removed after use. Drug release rate is however more accurate with insoluble inserts compared to soluble and biodegradable inserts which show larger variability between different patients due to different tear turnover rates and metabolic enzyme concentrations. (Furrer et al. 2008, Rathore & Nema 2009; Saettone & Salminen 1995; Del Amo & Urtti 2008)

Drug delivery can be enhanced also by encapsulating drugs into transport vessels such as nanoparticles and liposomes (presented in Figure 9). Liposomes are structurally similar to phospholipid membranes and thus facilitate drug absorption across cell membranes by coming into close contact with membrane surface. (Furrer et al. 2008; Xu et al. 2013) The aqueous inner of the liposome can act as reservoir for hydrophilic drugs whereas surrounding lipid bilayer can uptake hydrophobic drugs (Xu et al. 2013).

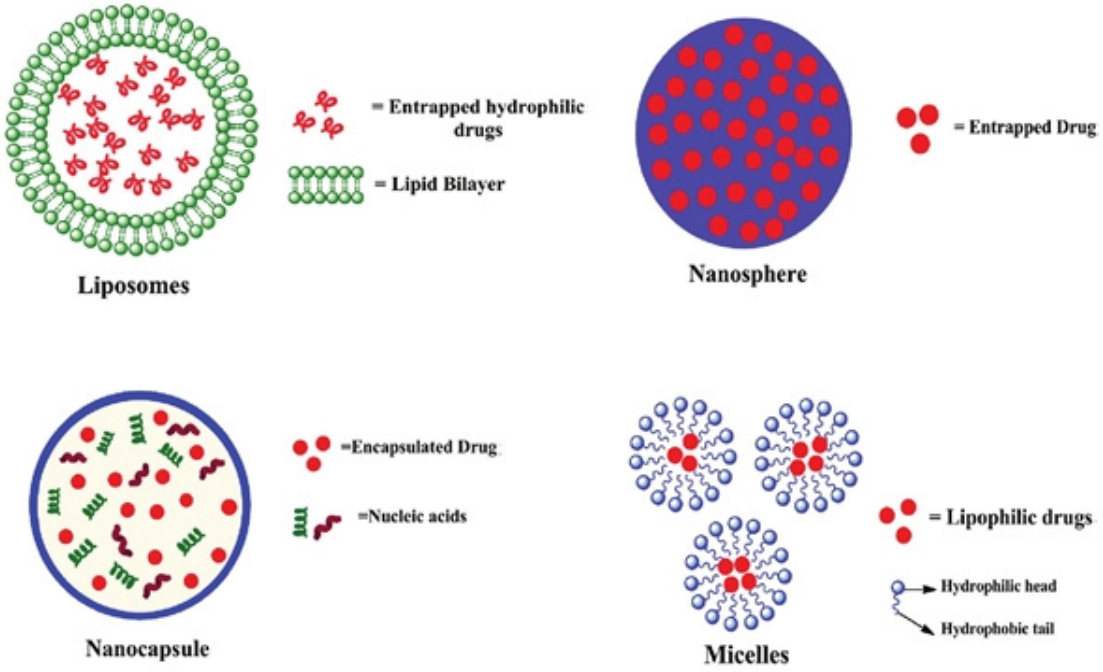

Figure 9. *Schematic presentation of some transport vessels used in ocular drug delivery. Modified from ref. (Xu et al. 2013)*

Nanoparticles are primarily polymer based delivery systems. Drugs can be either enclosed inside the nanoparticle (nanocapsule) or distributed throughout the polymeric matrix (nanosphere). Polymers with polar or ionized groups have electrostatic interaction with the negatively charged surface of cornea which increases the corneal contact time. Nanoparticles are more stable than liposomes and due to their small size they irritate ocular surface less. (Calvo et al. 1996; Monem et al. 2000; Smart 2005; Diebold et al. 2007; Xu et al. 2013)

Preservatives are added to ophthalmic drug products primarily to inhibit microbial contamination, but as a side effect, they have been reported to promote ocular absorption (Freeman & Kahook 2009). Various penetration enhancers such as benzalkonium chloride (BAK), ethylenediaminetetraacetic acid (EDTA), non-ionic surfactants and bile salts have been investigated for their potential in improving ocular drug absorption. Amphiphilic surfactants can be easily diffused into the cell membrane. As the cell membrane gets saturated with these surfactants, they form micelles (see Figure 9) which affect membrane composition and solubilization. This process increases the transcellular transport of the active drug. Widely used preservative and cationic surfactant, BAK increases the solubility of hydrophobic drugs. BAK also has a high affinity for membrane proteins. It accumulates easily in corneal epithelium which benefits it as an absorption enhancer but can also lead to unwanted toxicity. Also bile salts can alter the integrity of the cell membrane and its lipid composition. This affects the rheological properties of the membrane and thus, enhances drug permeability. (Mitra 2013; Grass et al. 1985; Kaur & Smitha 2002; Marsh & Maurice 1971; Morimoto et al. 1987; Gasset et al. 1974) Chelators, such as EDTA, can increase paracellular drug absorption by loosening the tight epithelial junctions via calcium chelation (Tomita et al. 1996).

Absorption rate can be affected by chancing the pH or the osmolality of the drug solution. Drug's lipophilicity and solubility are highly dependent on the ionization stage of the drug which can be altered by adjusting formulation pH. Eye can tolerate slight moderation of pH. However, adjusting pH below 6 or over 8 causes discomfort and enhances lacrimation which increases drug loss. (Furrer et al. 2008; Trolle-Lassen 1958) A large deviation from neutrality damages ocular tissues. Thus, pH values under 2 and over 11,5 are not acceptable in topical formulations. (Hurley et al. 1993) The natural osmolality of the tears is about 330 mOsm, but eye can tolerate slightly hypertonic and hypotonic solutions between 100 mOsm and 400 mOsm. Hypotonic solutions can be used to increase the absorption of the hydrophilic drugs due to solvent drag effect. (Chun et al. 2008)

Drug absorption can be enhanced also by using high permeability prodrugs. Prodrugs are pharmacologically inactive derivatives of parent drug compounds which have increased permeability and/or solubility compared to the parent drugs. They can be

converted into active parent drugs after passing through the membranes. For example lipophilic esters can be used as prodrugs since they hydrolyse chemically to the parent alcohol and carboxylic acid. There are also prodrugs which can be converted into active drugs by enzymes such as esterases, ketone reductases or peptidases. (Furrer et al. 2008; Järvinen & Järvinen 1996; Lee & Bundgaard 1992; Tammara & Crider 1996)

5. MODELS FOR THE EVALUATION OF OCULAR DRUG PERMEATION

5.1. Pharmacokinetic absorption studies in human

The best model for the evaluation of drug absorption in man is no doubt a man (Davis & Wilding 2001). Therefore absorption kinetics must be studied in human volunteers at some point of the drug development process (Dahiya et al. 2007). The rate and extent to which an active drug compound is absorbed from a drug product and becomes available at the site of drug action is called bioavailability. The bioavailability of systemic drugs is usually determined by measuring the concentration of an active substance in plasma as a function of time. (Chow 2014) Ocular pharmacokinetic studies in humans are unfortunately much more complicated since plasma level analysis does not usually correlate with the intraocular concentration and the ocular bioavailability of topically administrated drugs. However plasma level analysis might be useful if the systemic absorption of ophthalmic drug may cause some undesirable side effects. (Furrer et al. 2008)

Drug concentration in the aqueous and vitreous humor can be determined from patients undergoing surgical procedures such as cataract surgery (Koch et al. 2005) or vitrectomy (Puustjärvi et al. 2006). These methods are very invasive and hence ethically doubtful. Therefore, it is preferable to estimate the extent and rate of drug absorption from physiological responses. For example changes in intraocular pressure, miosis, mydriasis induced myopia or bactericidal activity can be easily measured and converted into bioavailability data. (Shell 1982; Furrer et al. 2008) Other non-invasive observation methods are gamma scintigraphy and fluorescent probes (Furrer et al. 2008). For example the corneal contact time of drugs in different formulations can be measured by gamma scintigraphy (Meseguer et al. 1996). Moreover pre-corneal contact time can be studied by measuring drug levels in tear (Walters et al. 2010). Tear sampling with pipette can however induce excessive blinking and tear production and therefore induce a bias in the results. Additionally, if drug formulation does not mix rapidly with the tear film, sampling even a small part of the formulation itself would lead to an overestimation. (Furrer et al. 2008)

5.2. Animal and tissue based models

As described in previous chapter, the pharmacokinetic studies of ophthalmic drugs are typically invasive and therefore not suitable for *in vivo* human trials. Thus, animal testing is an essential part of the drug development before clinical human trials. Unfortunately studying ocular absorption with animals requires usually sacrificing them. After drug administration, eyes must be cut apart to measure drug concentrations in different sections of the eye. (Liu et al. 2010)

Albino rabbit is the most commonly used animal model in ocular pharmacokinetic studies. Rabbits are relatively inexpensive and easy to handle. Rabbit's eye has rather large ocular surface area. There is also important background literature on the ocular effects of the chemicals in rabbits. The rabbit eye and the human eye have quite similar cornea and aqueous humor composition. However, there are also some anatomical and physiological differences between human and rabbit eye. Rabbit has a third eyelid and no well-defined Bowman's membrane. The blinking rate in rabbits is also much lower than in humans, which decreases precorneal drainage of topically applied drug solutions. (Plazonnet 2002)

Ocular drug permeability can be estimated also *ex vivo* by preparing fresh excised animal tissues and placing them in an appropriate diffusion chamber. Franz diffusion cells and Ussing chambers are the most commonly used devices to study corneal absorption and penetration (Figure 10). These systems consist of a donor and a receptor chambers filled with physiological buffer solutions, separated by the studied tissue such as cornea or sclera. Drug solution is applied to the donor chamber and the amount of drug crossing the tissue is analyzed at relevant time points from the receptor chamber. Chambers are surrounded by water jackets to maintain constant biological temperature. The viability of the tissues can be prolonged by continuous gas flow of oxygen and carbon dioxide. Solutions can be stirred by magnetic stirrer or by gas lift. (Agarwal & Rupenthal 2016)

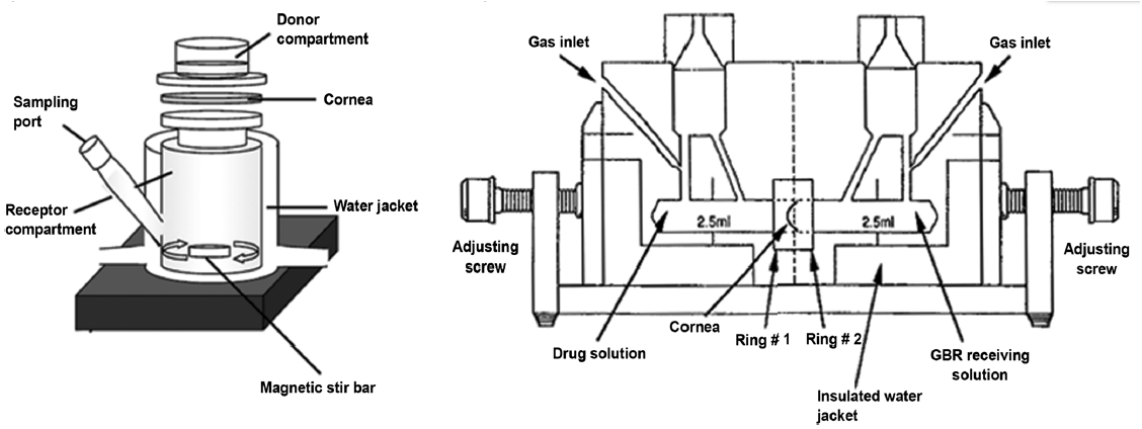

Figure 10. *Franz diffusion cell on the left and Ussing chamber on the right are the most commonly used devices to study drug permeability across tissues. (Agarwal & Rupenthal 2016, modified from ref. Todo et al. 2013 and Evitts 1990)*

Rabbit, porcine and bovine corneas are the most commonly used tissues for *ex vivo* permeability modelling. Human corneas cannot be used for permeation studies since donated human corneas are reserved only for transplant purposes. Compared to the *in vivo* animal trials these tissue based permeation studies are more ethical. They are especially useful in early drug formulation development as long as inter-species variation is properly taken into account and diffusion cell assembly simulates physiological conditions. (Agarwal & Rupenthal 2016)

5.3. Cell culture models

For both ethical and economic reasons, there is pressure to develop and validate cheaper and ethically more acceptable new *in vitro* test methods in order to replace or reduce animal testing. It is actually required that animal experimentation should never be performed when an alternative experiment method exists (Council Directive 86/609/EEC). Suitable *in vitro* cell culture models can reduce the need for animal testing. Typically these cell culture models are prepared by culturing harvested cells in appropriate growth medium and placed on a permeable filter. Drug permeability can be then measured by diffusion chambers described earlier in Chapter 5.2. (Agarwal & Rupenthal 2016)

Generally there are three different types of cell culture models: primary cell cultures, immortalized cell lines and reconstructed whole cornea tissue cultures. Primary cell cultures are prepared by isolating the cells straight from the tissue of interest. Since corneal epithelium is the main limiting barrier of ocular drug absorption, corneal epithelial cells are most commonly studied. Cell cultures can be prepared either from animal or human corneal epithelial (HCE) cell tissues, but due to the slower cell proliferation rate and the finite availability of human tissue, rabbit and rat tissues are more commonly used. Primary cell cultures typically consist of 5-6 cell layers. They are relatively cheap and easy to prepare, but the cultivation process is time-consuming and primary cell cultures have limited lifespan, because normal cells do not divide infinitely but die over time. (Agarwal & Rupenthal 2016; Toropainen 2007)

Immortalized cell lines however are capable of infinite cell proliferation due to the mutations. These cell lines can be obtained from tumors or modified from normal primary cells by chemicals or viral vectors that activate the mechanism for maintaining telomere length. The main advantage of immortalized cell lines is that the same cell line can be utilized over and over again which guarantees a level of continuity that primary cells cannot provide. However, the longer these cell lines are cultured the more likely they develop new mutations and chromosomal abnormalities which can alter for example enzyme expression and growth characteristics of cells. (Agarwal & Rupenthal 2016; Toropainen 2007) Both primary cell cultures and immortalized cell lines have shown promising results in permeation studies (Agarwal & Rupenthal 2016). Especially

HCE-T cell line simulates well human cornea and C-HCE cell line has shown good correlation with excised rabbit corneas (Toropainen et al. 2001; Xiang et al. 2009).

The main limitation for both primary and immortalized cell culture models is that the epithelial cell lines do not represent the whole cornea but only the epithelium. Reconstructed whole cornea tissue cultures however contain all three cell types of the cornea. Epithelial, stromal and endothelial cells are cultivated above each other. Three-layered construction mimics the morphology and the physiology of human cornea more accurately than epithelial layer alone. (Agarwal & Rupenthal 2016; Toropainen 2007) It has been reported to be very applicable model for corneal irritation and toxicity estimation (Griffith et al. 1999). The whole corneal construct models have been used in permeability studies as well. Drug permeation across whole corneal construct model has been reported to be faster than trough excised corneas, but model show the same permeation rank order for the different drug formulations, which indicates that the barrier functions of the whole cornea tissue culture and the excised cornea are quite similar (Tegtmeyer et al. 2001). Unfortunately these three-dimensional cornea constructs have not been extensively studied. Therefore there is no evidence that these more complex models are more beneficial compared to simpler epithelial models. (Rönkkö et al. 2016)

One of the greatest challenge for cell culture models in the lack of standardization. Interlaboratory data comparison is difficult due to the differences in experimental conditions, cell sources and cell culturing methods. The use of reference compounds in permeation studies and transepithelial electrical resistance (TER) measurements of the cell cultures support standardization between laboratories. (Mälkiä et al. 2004)

5.4. Artificial membranes and PAMPA

Permeability measurements that utilize artificial lipid membranes can be classified in-between biological and physicochemical methods. PAMPA (parallel artificial membrane permeability assay) is a widely used high-throughput technique developed for the evaluation of the passive transcellular permeability of drugs. It was first introduced by Kansy et al. 1998. In this system an artificial membrane immobilized on a 96-well filter plate is placed between a donor and acceptor compartments (Figure 11). In the beginning of the test, buffer solution containing the analytes is introduced in the donor compartment and a fresh buffer solution is placed in the acceptor compartment. Two compartments are then assembled into a “sandwich-like” configuration and incubated for a determined time, after which the analyte concentrations in both the donor and acceptor compartments are measured and compared with initial concentration in the donor compartment. Concentrations can be measured by UV spectrophotometry using a 96-well microplate photometer. (Kansy et al. 1998; Kerns & Di 2004)

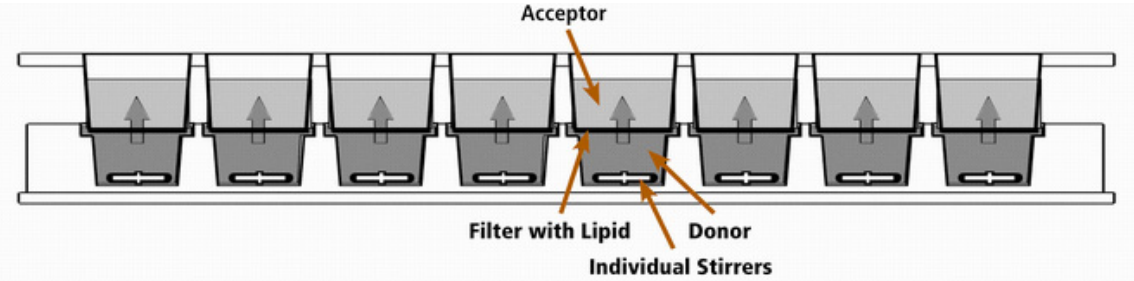

Figure 11. *Parallel artificial membrane permeability assay. (Pion-inc 2018)*

Artificial membrane can be formed by impregnating the supporting filter plate with organic solution containing phospholipids. Therefore this membrane is totally artificial and does not contain any inter-cellular pores or active transport system. (Kansy et al. 1998) Depending on the lipid composition of the artificial membrane, different barrier tissues can be simulated. Because most drugs are administered orally, intestinal absorption has been studied most extensively. In the original PAMPA method, artificial membrane was created by immersing a lipophilic microfilter in phosphatidylcholine (egg lecithin) dissolved in *n*-dodecane, as mammalian membranes are predominantly composed of phosphatidylcholine (Kansy et al. 1998). Cholesterol has often been added to previous mixture due to its significance in natural mammalian membranes (Sugano et al. 2001). It has been also demonstrated that some solvents alone, such as *n*-hexadecane can model the membrane permeability sufficiently (Wohnsland & Faller 2001).

PAMPA models have been developed also to mimic blood-brain barrier (BBB) and skin permeability. Artificial membrane containing porcine brain tissue extract dissolved in dodecane is able to model accurately compounds which pass the BBB and those which poorly penetrate the BBB. (Di et al. 2003) Membrane consisting of isopropyl myristate and silicone oil is able to mimic the human stratum corneum and can be used to predict passive human skin permeability (Ottaviani et al. 2006). Artificial membrane containing corneal epithelium membrane lipid mixtures is surely possible to develop, but so far PAMPA models have not been studied specifically for ocular permeation. Therefore, true benefits of PAMPA in comparison with cell culture models are still open in ophthalmic drug development. PAMPA however is a high-throughput technique that allows rapid determination of permeability profiles for different formulations over wide pH range, which is more difficult to obtain from cell culture experiments (Wohnsland & Faller 2001).

6. PREDICTING DRUG PERMEATION BY PHYSICOCHEMICAL PARAMETERS

As has been demonstrated earlier in Chapter 3, physicochemical properties such as the molecular size, ionization constants, lipophilicity, solubility and hydrogen bonding property of pharmaceutical products have a huge impact on membrane permeability and bioavailability. Thus, these properties can be used to predict drug absorption. Physicochemical analysis methods are attractive because they predict passive drug permeation reproducibly and efficiently. These methods also have great high throughput potential and they require less resources and manpower compared with many other *in vitro* permeability prediction methods. However, these methods do not take into account physiological conditions or any specific interactions between drugs and membrane. (Balimane et al. 2000)

6.1. Experimental determination of ionization constant

The ionization constant pK_a of the drugs are traditionally measured by potentiometric method, but also UV spectrophotometry, capillary electrophoresis and liquid chromatography can be used. The determination of pK_a by reversed phase high performance liquid chromatography is introduced more detailed in chapter 7.3.

6.1.1. Potentiometric titration

In potentiometric titration a precisely known volume of standardized strong acid or base solution is added to a stirred solution of an ionizable analyte compound and the pH of the solution is continuously measured with a standardized electrode which has been calibrated beforehand by blank titration. The pK_a is determined from the titration curve (Figure 12) at the point where the half of the compound is in the ionized form and half is in the unionized form. At this point pH is equal to pK_a. (Avdeef & Testa 2002)

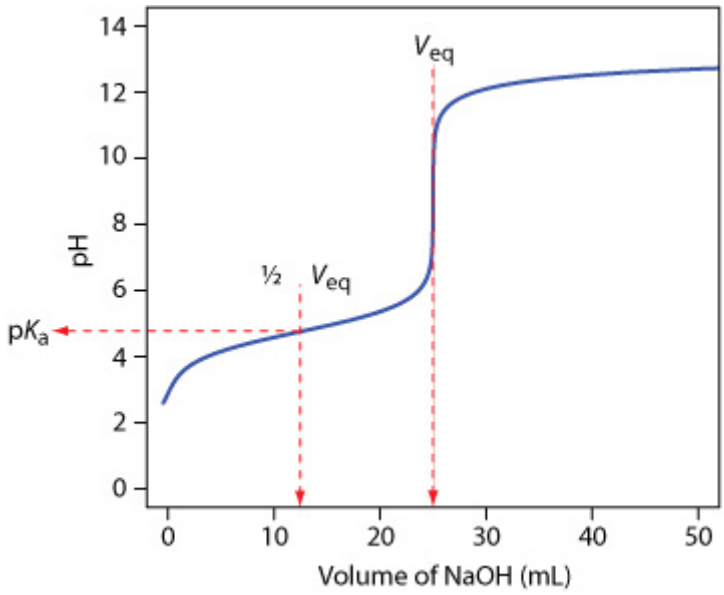

Figure 12. *Titration curve of weak acid with strong base. pK_a can be determined from the titration curve at the point where half of the compound is in the ionized form and half is in the unionized form. At this point pH is equal to pK_a. (Asdlib2018)*

Since pK_a depends on both temperature and ionic constant, ionic strength should be adjusted to physiologically relevant 0,15 M by using NaCl or KCl and the temperature must be kept constant at 25 °C. If the aqueous solubility of the analyte compound is insufficient, potentiometric titrations are performed at different co-solvent concentrations and the aqueous pK_a value is deduced by extrapolating the pK_a value to zero co-solvent concentration. Titrations can be performed manually, but also automated high-throughput commercial instruments has been developed (e.g. the Sirius GLpKa from Sirius Analytical Instruments). This automated technique takes 30-60 minutes per titration and requires a milligram amount of analyte. (Avdeef & Testa 2002, Kerns 2001)

6.1.2. Spectrophotometric pK_a determination

Spectrophotometric pK_a determination is based on the change in absorption of chromophores that are close to the ionization centers of the compounds. Ionization constants can be determined from a plot of absorbance as a function of pH. (Henchoz et al. 2009) Multiwavelength spectrophotometric titration method combines diode array detector, automated pH titrator and elaborate data treatment. With this method even multiprotic compound with overlapping pK_a values can be characterized. (Allen et al. 1998) For a high through-put demand a spectral gradient analysis (SGA) method has been developed and commercialized. In SGA technique, samples are delivered to the instrument in 96-well plates, after which solutions are automatically diluted and injected into a mobile phase that passes through a diode array UV detector. Mobile phase composition is altered continuously by mixing varying amount acidic and basic buffers

to create linear pH gradient over a period of 2 minutes. The determination of pK_a values of a compound takes only 4 minutes. Thus, the trough-put of this instrument is over 240 compounds per day. Since spectrophotometric determination is usually more sensitive than potentiometric method, sample consumption is lesser. However, this technique can be utilized only for compounds having a measurable pH dependent UV chromophore. (Kerns 2001; Box et al. 2000 and 2003]

6.1.3. Capillary zone electrophoresis

Ionization constants can be determined also by capillary zone electrophoresis (CZE). CZE is a chromatographic separation technique in which analytes are dissolved in buffer solution and separated in narrow fused-silica capillaries under the applied electrical field (Figure 13). The ends of the capillary are immersed into reservoirs filled with background electrolyte (BGE) solutions, which also contains electrodes. The migration of the analytes is initiated and maintained by a constant electric field created by high voltage supply. Due to the electro osmotic flow (EOF) all ions are pulled through the capillary in same direction. The analytes separate as they migrate due to their electrophoretic mobility. (Skoog et al. 2007; Piaggio & Deiber 2003)

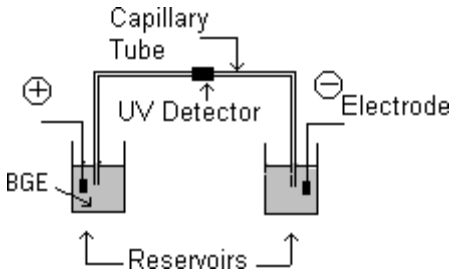

Figure 13. *Schematic presentation of the capillary electrophoresis system. (Piaggio & Deiber 2003)*

To determine the pK_a value of a compound, the effective mobility μ_{eff} of the compound is measured using mobile phases buffered at different pH values. The ratio between ionized and unionized depends on the pH. Because electrophoretic mobility is proportional to charge, the mobility of the compound is also pH dependent. (Kerns 2001) Effective mobility at different pH values can be calculated as:

$$\mu_{eff} = \frac{L_{eff} \cdot L_{tot}}{U} \cdot \left(\frac{1}{t_a} - \frac{1}{t_{EOF}} \right) \tag{9}$$

where t_a and t_{EOF} are the migration times of the analyte and the neutral marker compound that are carried through the capillary by the EOF, U is the applied voltage, L_{tot} is the total capillary length and L_{eff} is the effective capillary length. The pH depended variation of EOF can be corrected by using a neutral marker. The pK_a values

are obtained by fitting μ_{eff} as a function of pH to a suitable model for the number of ionizable groups. (Henchoz et al. 2009; Poole et al. 2004)

Although CZE has not been used as widely as potentiometric or spectrophotometric techniques to determine pK_a values, it has several advances compared to them. Sample consumption is very low (1-50 nl sample at concentrations of 10-500 μM), instrumentation is highly automated, impure samples can be measured, knowing the sample concentration is unnecessary since only mobilities are used in calculations and resolving power is excellent. (Henchoz et al. 2009; Poole et al. 2004) Multiprotic compounds having even seven ionization groups have been successfully characterized (Ishihama et al. 1994). This technique is also universal, because CZE can be combined with several different detection systems. The photodiode array is however the most commonly used detector for routine pK_a measurements. (Poole et al. 2004)

There are however also some drawbacks considering CZE. Major challenges with CZE method are the need of multiple experiment points at different pH values and the long migration times at low pH due to the limited EOF. To increase the throughput, dynamic coatings can be used create a high EOF. Migration times can be decreased also by using high external pressure or vacuum. For this purpose, pressure-assistant capillary electrophoresis (PACE) and vacuum-assisted 96-channel capillary electrophoresis has been developed. Choice of the buffer, applied voltage and the detection technique must be considered carefully to obtain reliable pK_a values by CZE (Henchoz et al. 2009).

6.2. Determination of lipophilicity by octanol-water partition coefficient

As has been told earlier in Chapter 3.3 the octanol-water partition coefficient (P_{ow}) is the most widely used measure of the lipophilicity of drugs (Mälkiä et al. 2004). There are however numerous different methods to measure and estimate P_{ow}. Octanol-water partition coefficient can be directly measured by traditional shake-flask method or by slow stirring method. It can be also estimated by theoretical calculations or by indirect experimental methods such as potentiometric titration or reversed phase high performance liquid chromatography (RP-HPLC). (Kah & Brown 2008) Chromatographic methods will be described separately later in Chapter 7.4 while the other methods are presented forthwith.

Octanol-water partition coefficient is traditionally measured by shake-flask method. This method is appropriate for non-ionized compounds having log P_{ow} in range of -2-4. Briefly, an analyte compound is dissolved in either n-octanol or water depending on its solubility, the remaining solvent is added to previous solution and the analyte-octanol-water mixture is shaken or stirred until equilibrium is reached. The organic and aqueous

phases are then separated, usually by centrifugation, and the concentration of the analyte compound in both phases is measured. (OECD 1995) P_{ow} can be then calculated from following equation:

$$P_{ow} = \frac{c_o}{c_w} \times \frac{V_w}{V_o} \tag{10}$$

where c_o and c_w are the analyte concentrations in octanol and water phases and V_o and V_w are the solvent volumes respectively (Gynther et al. 2003). Concentration determinations are usually performed by spectrophotometry, gas chromatography or high performance liquid chromatography. It must be calculated beforehand that the final concentration of analyte in each phase will be high enough to be analyzed by chosen analysis method, but still be less than 0,01 M. (OECD 1995) Basically this procedure is very simple, but in reality it is messy, time-consuming and requires relatively large amounts of solute (Mälkiä et al. 2004). Before a partition coefficient can be determined, octanol and water phases must be mutually saturated at the temperature of the experiment. Saturation can be achieved by shaking two large stock bottles, one containing n-octanol and a sufficient quantity of distilled water, and the other containing distilled water and a sufficient quantity of n-octanol, for 24 hours, after which bottles are let to stand long enough to allow the phases to separate. Accurate and reliable partition coefficients can be obtained by making duplicate determinations under three different test conditions by varying the solvent volumes. The determined log P_{ow} values should fall within a range of ± 0,3 log units. Due to the formation of octanol emulsions in water, substances with high lipophilicity (log P_{ow} > 5) cannot be determined by this method. (OECD 1995 and 2004)

Slow-stirring method follows the same principle as shake-flask method. However, in this method the two phases are equilibrated under very slow stirring to avoid emulsion formation. Typical phase ratios used are 20-50 ml of n-octanol and 950-980 ml of water, total volume being a liter. Log P_{ow} values up to 8.2 have been accurately determined (De Bruijn et al. 1989). Therefore, slow-stirring method is suitable for highly lipophilic compounds that cannot be measured by shake-flask method. The whole procedure is however time consuming. Sampling of compound with log P_{ow} estimate less than five takes two to three days, but for a compound with log P_{ow} 8.23 even 144 hours equilibrium time was necessary. (OECD Proposal)

For ionizable compounds log P_{ow} values can be determined by potentiometric titration. This technique is similar to the potentiometric pK_a measurement described earlier in Chapter 6.1.1. P_{ow} determination however consists of two linked potentiometric titrations. Typically, a pre-acidified solution of weak acid is titrated first with standardized base to some appropriately high pH to measure pK_a value in water phase. Octanol is then added and the mixture is titrated back to the starting pH to measure the

apparent pK_a value (p$_o K_a$) of test compound in two-phase system. If the test compound partitions into the octanol phase, pK_a values derived from the aqueous and the dual-phase titration curves are different. A large difference between pK_a and p$_o K_a$ indicates a large value of log P_{ow}. Depending on the nature of ionizable analyte compound, different equations can be used to derive P_{ow} from pK_a and p$_o K_a$ values. For example, the log P_{ow} value for neutral species of monoprotic compound can be calculated using the following equation:

$$P_{ow} = \frac{10^{|poKa - pKa|} - 1}{r} \tag{11}$$

where r is the volume ration $V_{n\text{-octanol}}/V_{water}$. Before any measurements it is useful to have an estimate of P_{ow} value to choose optimum volume ratio r used in titrations. The basic principle is that the lower the estimated log P_{ow} values the larger the values of r are required for measurement. In order to obtain reliable P_{ow} values, at least three determinations should be made. The determined log P_{ow} values should fall within a range of ± 0,1 log units. The main advantage of this method is that it provides a pH profile of partitioning. Determination of log P_{ow} for ionizable and multiprotic compounds is appropriate in the range -2 to 7. (OECD 2000) Titrations can be performed manually, but the entire operation of titrations and calculations has also been automated. Previously described high-throughput commercial instrument (Sirius GLpKa from Sirius Analytical Instruments) can be used to determine both pK_a, p$_o K_a$ and log P_{ow} values.

Filter probe method is similar to the potentiometric method except that the partitioning is determined by spectroscopy instead of potentiometry. An aqueous solution of analyte is placed in a reaction vessel and circulated through an UV flow cell. The absorbance of the aqueous solution is measured both before and after addition of octanol. If the analyte partitions into the octanol phase, the absorbance of the aqueous solution is decreased. To prevent any octanol from passing through to the detector a solvent inlet filter is used. Log D profiles can be obtained by performing the experiments at different pH values. The method is rapid and reliable but cannot be applied for molecules having log P values less than 0.2. It is also required that the analyte compound has an isobestic point in its UV spectrum. Isobestic point is a specific wavelength at which the absorption spectra of both ionized and neutral species cross each other. (Kah & Brown 2008; Niazi 2007)

Although octanol-water partition coefficient is the most extensively used measure of the lipophilicity of drugs, it has been criticized for supporting hydrogen bonding which can lead to permeability overestimation for drugs which are capable of forming hydrogen bonds (Mälkiä et al. 2004). The hydroxyl group of *n*-octanol can act both as a hydrogen donor and an acceptor. However, different types of cell membranes have distinct

hydrogen bonding properties. Thus, alternative solvents such as chloroform, dibutyl ether, cyclohexane, dodecane and 1,2-dicloroethane have been proposed to imitate different kind of membranes. Octanol is the most suitable solvent when target membranes contain amphiprotic groups, whereas chloroforms suits well for the membranes containing mainly hydrogen donors and dibutyl ether for the membranes containing mainly hydrogen acceptors. If the target membrane contains neither hydrogen donors nor acceptors, alkanes such as cyclohexane and dodecane can imitate the membrane barrier. (Hartmann & Schmitt 2004; Giaginis & Tsantili-Kakoulidou 2008) Since many compounds have low alkane solubility, 1,2-dicloroethane has been proposed to replace alkanes in partitioning experiments (Steyaert et al. 1997).

In addition, liposome-water systems have been used to model drug partition into biological membranes. Especially, for charged compounds octanol-water system often fails to model electrostatic interactions between the drug and the membrane (Esteves et al. 2013). Liposomes that are converted from phospholipids, usually from phosphatidylcholines, can simulate the electrostatics of the lipid bilayer more authentically (van Balen et al. 2004). Despite the weaknesses of octanol-water system and the increasing popularity of liposomal partitioning determination, *n*-octanol has remained as the main solvent for partition experiments. Due to the huge log P_{ow} databases it can be considered as the reference system for lipophilicity estimation (Giaginis & Tsantili-Kakoulidou 2008).

7. LIQUID CHROMATOGRAPHY METHODS IN DRUG PERMEATION STUDIES

7.1. High performance liquid chromatography as a separation method

Chromatography is a separation method, in which components to be separated are distributed between two immiscible phases, stationary and mobile phase. Components of the mixture are separated as they travel in mobile phase that flows through the stationary phase. Separation is based on differences in migration rates along sample components. It occurs due to the different interaction of each sample component with the stationary and mobile phases. The stationary phase can be either solid or liquid, while the mobile phase is usually either liquid or gas. The techniques are named after the mobile phase as gas chromatography (GC) or liquid chromatography (LC).

High performance liquid chromatography (HPLC) is a form of liquid chromatography in which analytes are dissolved in liquid mobile phase called eluent vie sample injector, and the solution is forced to pass through a chromatography column containing stationary phase under high pressure at certain flow rate. Many systems also include an oven for the temperature control of the column. Compounds are separated, since they travel at different individual speeds through the column. Separation efficiency depends both on the choices of mobile and stationary phases. Separation is monitored with flow-rate detector and chromatographic data is presented as a graph of detector response against elution time, which is called chromatogram. Examples of chromatograms are presented in Appendix 1. As a result, HPLC has the ability separate, identity and quantitate a wide variety of chemicals and their mixtures. (Harris 2007; Kazakevich & LoBrutto 2007)

From the chromatogram several important parameter can be deduced to characterize the efficiency of separation and column efficiency. The most important parameter in the chromatographic measurement is the time taken for a particular compound to travel through the column to the detector, called retention time, t_r. This time is measured between the injection point and the maximum detector response of correspondent compound. The retention times can be directly measured from the chromatogram. Retention times are highly dependent on the geometry of the column, nature of the stationary and mobile phases, temperature and flow rate. [Harris 2007; Kazakevich & LoBrutto 2007)

The more universal retention parameter is a retention (capacity) factor *k*, defined as:

$$k = \frac{t_r - t_0}{t_0} \quad (12)$$

where t_o is the retention time of an unretained component and t_r is the retention time of the analyte respectively. Retention factor is independent on mobile phase flow rate and geometry of the column. Ideally, if the retention of the same sample is measured with same stationary and mobile phase by different instruments, theoretically the retention factors should be identical. This allows comparison of the results between different instruments and laboratories. [Harris 2007; Kazakevich & LoBrutto 2007)

7.2. Classification of separation methods in HPLC

Liquid chromatographic methods can be classified depending on the interaction mechanism between solute and stationary phase. In adsorption chromatography stationary phase is an absorbent and the separation is based on repeated absorption-desorption steps. The solute competes with the solvent for binding sites. The stronger the solute is absorbed the slower it passes through the column. In partition chromatography both stationary and mobile phases are liquids and the separation is based on the selective partition of solute between them. Ion-exchange chromatography utilizes the differences in ion-exchange properties of solutes, and the exclusion chromatography separates molecules by size. The most selective kind of chromatography, affinity chromatography is based on a specific interaction between an analyte molecule and a second molecule, for example an antibody immobilized on stationary phase. (Harris 2007)

Adsorption chromatography can be further divided into two different categories, normal-phase (NP) and reversed-phase (RP) chromatography. In normal-phase chromatography polar stationary phases, such as silica gel, and non-polar mobile phases are used. Hydrophilic solutes are retained stronger on the surface of the stationary phase than hydrophobic solutes. As opposite to NP-HPLC, in reversed-phase chromatography the polarities of mobile and stationary phases are reversed. The surface of the stationary phase is non-polar and mobile phase is polar. RP-HPLC is the most commonly used form of chromatography. (Harris 2007; Kazakevich & LoBrutto 2007)

Liquid chromatographic methods can be classified also by elution types. There are two different elution types: isocratic and gradient elution. In isocratic elution the eluent composition is kept constant during the whole analysis. In gradient elution, the eluent composition is steadily changed during the run. (Harris 2007)

7.3. HPLC method to determine pK_a

RP-HPLC can be used to determine the ionization constant of ionizable drugs. Method was first reported by Horvath et al (1977). The chromatographic retention of ionizable compound is highly dependent on the pK_a of the analyte and the pH of the mobile phase. The neutral forms of the compounds are well retained due to their high affinity to the lipophilic stationary phase. Ionized forms have shorter retention times because they are more soluble in polar mobile phase respectively. When the pH of the mobile phase is lower than the pK_a of a weak acid, the compound will be in neutral form. Thus weak acids will retain more at low pH than at high pH. Conversely weak basics are in neutral form and well retained when the pH is higher than the pK_a. (Horvath et al. 1977; Wiczling et al. 2004; Gynther et al. 2003)

Since the pH of the mobile phase affects the retention of ionizable compounds, pK_a of the compound can be determined by measuring the retention times of the drug compound at 8-10 different pH of the eluent and plotting the retention factor k as a function of pH. For weak acids the dependence of k and pH can be illustrated by following equation:

$$k = \frac{k_{HA} + k_{A^-} \times 10^{pH - pKa}}{1 + 10^{pH - pKa}} \tag{13}$$

where k_{HA} and k_{A^-} refer to the retention factors of the deprotonated and the protonated forms of acid respectively. At the point where pH equals to the pK_a value of the compound, Equation 13 can be simplified in form:

$$k = \frac{k_{HA} + k_{A^-}}{2} \tag{14}$$

Thus, pK_a value can be determined from the graph at the point where retention factor k is the average of the retention factors of unionized and ionized forms. Ideally, these isocratic measurements should be made in aqueous buffer solutions, but the retention times for neutral forms are typically so long in aqueous mobile phase that some organic solvent must be added to the mobile phase to speed up the elution. Unfortunately, the addition of organic solvent affects both the pH of the eluent and the ionization of weak acids and bases which complicates the estimation of pK_a values. Thus, pK_a values obtained by RP-HPLC are typically rough estimates. (Kazakevich & LoBrutto 2007; Horvath et al. 1977; Valkó 2016; Hardcastle & Jano 1998; Wiczling et al. 2004; Kaliszan et al. 2002)

7.4. HPLC methods to determine lipophilicity

Retention of a compound in RP-HPLC is mostly governed by its lipophilicity, since the retention time relates to the compound's distribution between the polar mobile phase

and nonpolar stationary phase. Retention factor k is equal to the ratio of the amounts of a sample component in the stationary and mobile phases respectively. Therefore directly measured retention factors are lipophilicity indices on their own. However retention factors can be also converted to log P_{ow} scale by using standards and correlation equations. (Avdeef & Testa 2002; IUPAC Gold Book)

Lipophilicity measurements can be performed in either isocratic or in gradient mode. Isocratic log k values are a relative scale of lipophilicity on their own. However log k values are usually extrapolated to pure water log k_w values to obtain more comparable lipophilicity indices. In order to do this, log k values are determined at various concentrations of organic solvent in mobile phase. There is a linear relationship between the volume fractions of organic solvent in mobile phase φ and the logarithmic retention factor log k:

$$\log k = -S\varphi + \log k_w \tag{15}$$

where S is the slope. Linearity holds optimally for organic solvent concentrations within the rage -0.5 < log k < 1.5. (Pallicer et al. 2010; Valkó 2012) Extrapolated pure water log k_w values can be also converted into more comparable octanol-water partition coefficients. The correlation requires calibrating the HPLC-system with at least six reference compounds for which both measured log k_w values and log P_{ow} values are known. If possible, reference substances should be structurally related to the test substance and at least one of the reference substances should have a log P_{ow} above and another a log P_{ow} below that of the test substance in order to avoid the need of extrapolation. (OECD 2004)

Another lipophilicity parameter, chromatographic hydrophobicity index (CHI) φ_0 can be obtained at the point where the line $\log k = -S\varphi + \log k_w$ intersect with the x-axis by the following expression:

$$\varphi_0 = \frac{\log k_w}{S} \tag{16}$$

This parameter, φ_0, is equal to the organic modifier fraction in the mobile phase at which the log k = 0. φ_0 varies from 0 to 100 for the most compounds. CHI values have shown relatively good correlation to log P_{ow} values even for structurally unrelated compound (Valkó et al. 1993, 1997 and 2012)

The main disadvantage of the isocratic CHI method is the need of several retention time measurements for the same compound which consumes time and resources. Therefore, fast gradient method has been developed to determine the CHI values. In this method

the gradient retention times of the reference and the analyte compounds are measured by applying the linear increase of the organic solvent fraction from 0 to 100 %. Due to high flow rates and short column lengths analyses are usually performed in less than 5 minutes. Acetonitrile is most commonly used as an organic solvent. Gradient retention times show good correlation with isocratic CHI φ_0 values because it can be assumed that the injected samples are absorbed to the stationary phase of the column and are not desorbed until the mobile phase composition reaches a certain sample specific organic phase concentration after which the compounds elute rapidly through the column. Thus, by a performing a simple linear regression, the measured gradient retention times t_r can be converted into the CHI values of the test and reference compounds after defining the coefficients A and B of following equation:

$$\mathrm{CHI} = At_\mathrm{r} + B \tag{17}$$

The CHI values of the reference compounds can be obtained from literature. For example GlaxoSmithKline has measured the CHI values for more than half a million compounds. (Valkó et al. 1997, 2012 and 2016)

7.5. Biomimetic chromatography

HPLC technique can be utilized to study also more specific biomimetic properties of the drugs such as phospholipophilicity and protein binding. In these applications stationary phases have been designed to imitate the biological environment. (Valkó 2016) Several methods have been developed to mimic the partitioning of the drugs into the phospholipid bilayers. Commercialized immobilized artificial membrane (IAM) chromatography utilizes stationary phases in which phosphatidylcholine lipids are covalently attached on silica resins to form ordered phospholipid surfaces (Pidgeon et al. 1995). Immobilized liposome chromatography (ILC) has similar idea, but in this technique lipids are bound to silica gel beads to form multi- or unilamellar vesicles (Liu et al. 2002). Biopartitioning micellar chromatography (BMC) uses common RP-HPLC stationary phases and surfactant is added to mobile phases to produce biomimetic micelles (Molero-Monfort et al. 2001; Escuder-Gilabert et al. 2004). The advantage of these methods is the ability to imitate the structure of biological membranes and therefore the results correlate generally well with biological permeation. (Valkó 2016)

Protein binding can heavily affect the bioavailability of the drugs, because proteins can act as reservoirs of the drugs. Drug's potential is therefore dependent on its affinity bind in proteins. (Trainor 2007; Schmidt et al. 2010) Drug-protein binding for at least two different plasma proteins has been characterized by HPLC. In these methods human serum albumin (HSA) or alpha-1-acid glycoprotein (AGP) have been immobilized on stationary phase by bonding them chemically to silica particles. Aqueous buffers have been typically used as mobile phase, but if studied compounds bind very strongly to the

protein stationary phase, 2-propanol can be used to increase eluent strength. However, high organic phase concentrations can denature protein stationary phases. Measured retention factors can be converted into percentage protein binding. (Valkó 2016; Vuignier et al. 2013)

7.6. Ion-pair chromatography

Ion-pair chromatography (IPC), also called as ion-interaction or solvent generated ion-exchange chromatography is a mode of HPLC method between ion-exchange and reversed phase chromatography. It can be performed on standard reversed phase columns, such as C18-columns, using ion-pair reagents in hydro-organic mobile phases. Ion pair reagents are usually amphiphilic molecules having a hydrophobic hydrocarbon chain and a charged head group. Addition of these ionic additives in mobile phase affects retention behavior of ionic or ionizable analytes. (García-Álvarez-Coque et al. 2017; Kazakevich & LoBrutto 2007) Thus, ion-pair chromatography could be utilized for example in revealing ion-pair formation between drugs and formulation excipients.

There are numerous competing theories for describing the retention mechanisms of IPC. In principle ion-pair formation can occur either in mobile phase or at the surface of stationary phase. Non-polar end of the amphiphilic counter-ion can be adsorbed on the hydrophobic stationary phase surface leaving the charged head group sticking out to the mobile phase. This creates a charged surface which attracts oppositely charged analyte molecules and increases their retention. On the other hand ion-pairing between ion-pair reagent and analyte can take place also in mobile phase after which this neutral complex is retained more strongly due to preferable partition into the stationary phase. In reality probably both mechanisms coexist. (Kazakevich & LoBrutto 2007; Moldoveanu & David 2013; García-Álvarez-Coque et al. 2017)

8. INTRODUCTION TO EXPERIMENTAL STUDY

Experimental part of the thesis was performed in the Quality Control and Analytical Development Laboratory of Santen Oy during September 2010 – January 2011. Physicochemical laboratory models have not been widely utilized for developing ophthalmic formulations in Santen Oy. Pre-clinical pharmacokinetic studies have been primarily based on *in vivo* studies in animal models. Furthermore formulation development had mainly relied on physicochemical parameters obtained from literature or supplied by ingredient manufactures. However, availability of this information is quite poor and parameters are often rough estimates. Thus, developing in-house physicochemical laboratory models to determine formulation parameters would improve the formulation development. Ideally simple laboratory models could explain or even predict the pharmacokinetic studies performed in animals.

The main idea for experimental study was to find out if common RP-HPLC instruments could be exploited in ophthalmic drug formulation development. As was demonstrated in theoretical part of this thesis, lipophilicity of a drug compounds can be estimated from its retention behavior in RP-HPLC. In this study, lipophilicity of five ophthalmic drug compounds was studied by common C18-HPLC system. For each compound, lipophilicity expressed as log k, was measured over a wide pH range to obtain pH-lipophilicity profiles which could be utilized while choosing the optimal formulation pH. It was also studied if some specific formulation additives affect the lipophilicity of these drug compounds.

Experimental part is divided into three different chapters. In the first chapter, lipophilicity of two commercial isopropyl ester prodrugs (**1e** and **2e)** and their biologically active metabolites (**1a** and **2a**) are studied by HPLC. Chapter consists of eluent optimization for further lipophilicity measurements and retention behavior studies over a wide eluent pH range. Moreover, it is studied if some specific interactions such as ion pairing, between these drug molecules and additive **X** could be revealed with a C18-HPLC-system by adding increasing amount of the additive **X** into the eluent.

In the second chapter, a modified shake-flask method is set up for determining direct log P_{ow} values of drugs at Santen Oy. Lipophilicity (log P_{ow}) of prodrug **2e** with and without additive **X** is directly measured by this method in order to compare results obtained in previous chapter by RP-HPLC method.

In the third chapter, usefulness of the C18-HPLC method for studying drug-additive interactions is put to the test. Method is validated by using common anti-glaucoma drug timolol and a sorbate additive, for which ion pairing had been previously verified by NMR (Higashiyama et al. 2007). Before ion pairing studies, the eluent composition is optimized for timolol. Additionally, the pH-lipophilicity profile of timolol is measured.

9. HPLC STUDIES OF ISOPROPYL ESTER PRODRUGS AND THEIR ACID FORMS

9.1. Background and purpose of the study

In this chapter, lipophilicity of two commercial isopropyl ester prodrugs (**1e** and **2e)** and their biologically active metabolites (**1a** and **2a**) are studied by HPLC. Chapter begins with eluent optimization and lipophilicity measurements, after which retention behavior is studied over a wide eluent pH range. Moreover, it is studied if some specific interactions such as ion pairing, between these drug molecules and an additive **X** could be revealed with a C18-HPLC-system by adding increasing amount of the additive **X** into the eluent.

Ophthalmic pharmaceuticals **1e** and **2e** are isopropyl ester prodrugs. They are hydrolyzed by esterases in the cornea to corresponding acids **1a** and **2a** which are biologically active. The prodrugs themselves are lipophilic, but the biologically active acid forms are hydrophilic. Due to the low water solubility, some solubilizing additives must be added to the aqueous eye drop formulations in order to dissolve the isopropyl ester prodrugs. Pharmacokinetic studies in animals have suggested that a common ophthalmic drug solubilizing additive **X** increases the bioavailability of prodrug **1e** by increasing its aqueous humor concentration after topical administration (Santen Oy). Additive **X** is a primary amine and exists in physiological pH in cationic form, while acid **1a** is assumed to exist in anionic form respectively. Thus, ion pair formation between these oppositely charged moieties has been suggested. It is assumed that ion-pair formation might increase the lipophilicity of acid **1a** and therefore explain its enhanced corneal permeability. On the other hand, additive **X** might have some other specific interaction with ester form which decreases the lipophilicity of prodrug **1e**. It also cannot be excluded that additive **X** affects the biological structure of corneal surface. Thus, the first aim of this study was to find out if assumed ion-pairing between acid form **1a** and additive **X** could be verified as an increased retention time by RP-HPLC instrument when adding additive **X** into the eluent.

Another isopropyl ester prodrug **2e** is structurally very similar with **1e**. It is as well hydrolyzed in the cornea to biologically active acid **2a**. Therefore it's possible that **2a** forms ion pair with additive **X** as well. Thus, another aim of the study was to find out if similar chemical interaction between additive **X** and acid **2a** can be observed by RP-HPLC as in case of acid **1a**.

9.2. Materials and methods

9.2.1. Buffer preparations

Acetate buffer solution (AcOH) was prepared by mixing desired volume of glacial acetic acid with Milli-Q water. Sodium phosphate buffer (SPB) solutions were prepared by dissolving required amount of disodium hydrogen phosphate dodecahydrate in Milli-Q water and adjusting the pH with 8,5 % phosphoric acid solution. Measurements in which drug–additive interactions were studied, additive was dissolved in buffer solution before pH adjustment. Modified Britton–Robinson buffer (Britton & Robinson 1931) was used as universal buffer for pH dependence studies. It was prepared by adding equal volumes of 1 M phosphoric acid, 1 M boric acid and 1 M acetic acid into Milli-Q water in order to bring the final concentration of each acid to 0,02 M. The desired pH was adjusted by addition of 1 M sodium hydroxide solution.

9.2.2. pH measurements

Buffer and HPLC eluent pHs were measured by Mettler Toledo MA 235 pH/Ion Analyzer. Electrode was calibrated against aqueous buffers using four-point calibration. Since there are different pH scales that are employed in pH measurement of HPLC mobile phases, henceforward measured pH values are represented as either ${}^{w}_{w}pH$ or ${}^{s}_{w}pH$ according to the IUPAC nomenclature (Inczédy et al. 1998). ${}^{w}_{w}pH$ refers to the pH value of an aqueous portion of the mobile phase before the addition of acetonitrile (MeCN) and ${}^{s}_{w}pH$ refers to the pH value of a hydro-organic mobile phase measured after the addition of acetonitrile respectively.

9.2.3. HPLC instrumentation and settings

HPLC analyses were performed with Waters 2695 Alliance Separations Module equipped with a Waters 2996 Photodiode Array Detector (Waters Corporation, US) using Empower2 Chromatography Data Software for instrument control and data acquisition and processing. Phenomenex-Gemini C18 column (150 x 2,1 mm i.d.; 3 µm) was chosen for analyses due to its low free silanol activity, extended pH stability (over pH range 1-12) and minimum eluent consumption. In most analyses column was equipped with a Phenomenex security guard column. Injection volume of the samples was 20 µl. Eluent flow rate was set at 0,15 ml/min while guard column was used and 0,20 ml/min without it respectively. Retention times t_r were measured at least duplicate and they were converted into retention factors k by applying Equation 12 in order to cancel out the effect of different flow rates. Void volume time t_0 was determined by uracil. All analyses were performed in room temperature and the column was stabilized for at least 30 minutes before analyses. The detection wavelength was set at 215 nm.

Before HPLC-studies aqueous mobile phases were filtrated trough 0,45 µm HATF mixed cellulose ester membrane filter (Millipore). Hydro organic mobile phases were filtrated trough 0,45 µm HVLP Durapore membrane filter (Millipore). Additionally, all mobile phases were degassed by ultrasonicator before use. Samples were dissolved in 20 % acetonitrile solutions. Sample concentrations are presented in Table 1.

Table 1. *Sample concentrations of prodrugs (**1e** and **2e**) and acid forms (**1a** and **2a**).*

	concentrations [µg/ml]			
	1a	**2a**	**1e**	**2e**
Mixed sample of all drugs (1a, 2a, 1e, 2e)	5,1	6,0	6,3	6,3
Mixed sample on acid forms (1a, 2a)	10,1	12,0		
Mixed sample of ester forms (1e, 2e)			12,6	12,5

9.3. Results and discussion

9.3.1. Method development and eluent optimization

Lipophilicity studies of two commercial isopropyl ester prodrugs (**1e** and **2e)** and their biologically active metabolites (**1a** and **2a**) began with HPLC method development. Eluent optimization was started by modification of an existing HPLC/MS/MS method (Santen Oy) of **2a** and **2e** (see eluent 1 in Table 2). As was assumed, all compounds were separated well ($R > 2$) and eluted in less than 9 minutes. However, since the aim of this study was to mimic ophthalmic drug formulations, eluent pH should be as close to neutral pH as possible. Therefore, acetic acid was replaced by sodium phosphate buffer ($^{w}_{w}pH$ 6,99) as an aqueous phase. The increase of eluent pH however decreased the retention of acid forms due to ionization (see eluent 2 in Table 2). Some peak tailing was also observed. Hydrophilic acid forms did not retain and separate enough despite that the eluent strength was decreased down to 40:60 MeCN/phosphate buffer. At the same time, such a weak eluent strength increased retention times and broadened peaks of more lipophilic ester forms. Additionally, eluent flow rate could not be increased due to the high back pressure of the HPLC instrument. Thus, simultaneous separation of all four components was not possible in reasonable run time by isocratic method. Applying gradient run on the other hand would have complicated subsequent studies and conclusions. Therefore separate isocratic methods were developed for acid and ester forms. The most suitable eluent compositions for both methods are highlighted in Table 2. Minimum criteria for these methods were good resolution ($R > 2$), reasonable run time (less than 10 minutes) and a sufficient retention.

Table 2. *Retention factors of **1a**, **2a**, **1e** and **2e** at different eluent compositions. The most suitable eluent compositions for both acid (**1a** and **2a**) and ester (**1e** and **2e**) forms are highlighted.*

		Retention factors, *k*				
Eluent	**pH**	**1a**	**2a**	**1e**	**2e**	**Resolution**
60 : 40 MeCN / 0,1 % AcOH	3,82 [1]	0,55	0,88	2,05	3,48	2,4 / 8,5 / 8,8
60 : 40 MeCN / SPB	6,99 [2]	0,16	0,26	1,95	3,29	< 1 / 11,4 / 8,1
50 : 50 MeCN / SPB	6,99 [2]	0,21	0,39	4,28	8,47	1,2 / 22,5 / 16,2
40 : 60 MeCN / SPB	6,99 [2]	0,38	0,79	14,18	33,90	2,8 / 41,3 / 23,8
70 : 30 MeCN / SPB	6,99 [2]			1,09	1,59	3,7
65 : 35 MeCN / SPB	6,99 [2]			1,42	2,22	5,4
60 : 40 MeCN / SPB	6,99 [2]			1,94	3,27	8,0
40 : 60 MeCN / SPB	6,99 [2]	0,39	0,79			2,8
35 : 65 MeCN / SPB	6,99 [2]	0,66	1,52			-
30 : 70 MeCN / SPB	6,99 [2]	1,33	3,52			10,5

9.3.2. Lipophilicity estimates

As a by-product of eluent optimization log *k* values were calculated from determined retention factors. The relationship between log *k* and the volume fractions of organic solvent acetonitrile in mobile phase φ appeared to be almost linear for ester forms **1e** and **2e** (see Figure 14) but for acid forms **1a** and **2a** linear approximation did not correlate as well (see Figure 15).

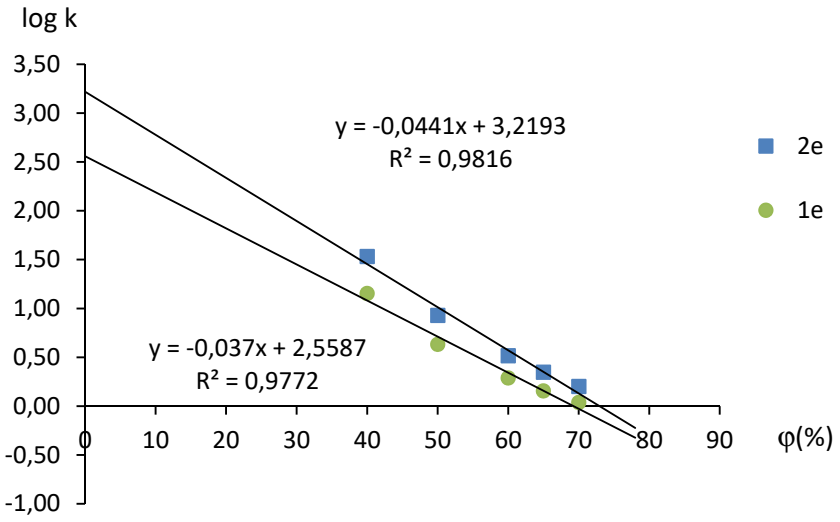

Figure 14. *Log k values of ester forms **1e** and **2e** as a function of volume fractions of organic solvent in mobile phase φ .*

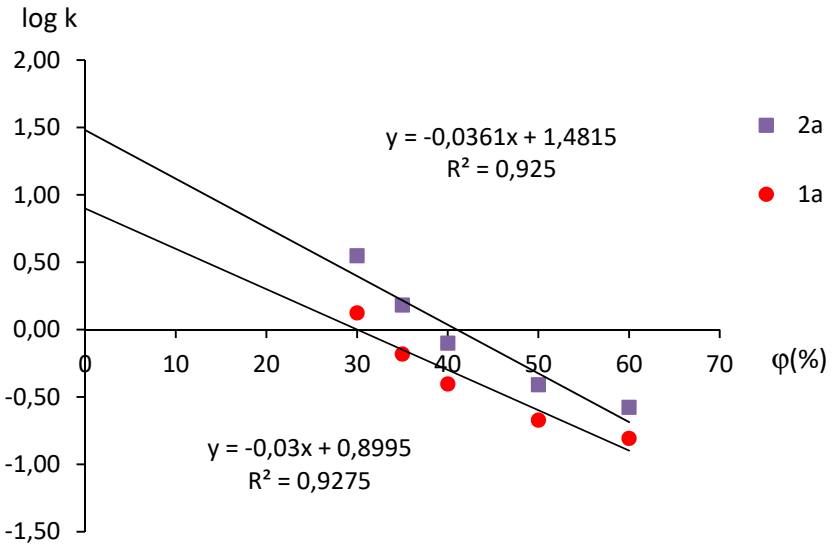

Figure 15. *Log k values of acid forms* ***1a*** *and* ***2a*** *as a function of volume fractions of organic solvent in mobile phase* φ *.*

As was represented in chapter 7.4, log k values are lipophilicity indices on their own, but log k values can be extrapolated to pure water log k_w values to obtain more comparable lipophilicity indices. Hence, log k_w values for each molecule were determined from their graphs at the points where the lines intersect with y-axis. Another lipophilicity parameter, chromatographic hydrophobicity index φ_0 was obtained at the points where the lines intersect with x-axis respectively (log k = 0). Extrapolated pure water log k_w and φ_0 values are represented in Table 3.

Table 3*. Extrapolated pure water log* k_w *and* φ_0 *values of* ***1a****,* ***2a****,* ***1e*** *and* ***2e****.*

	1a	**2a**	**1e**	**2e**
log k_w	0,90	1,48	2,56	3,22
φ_0	30,0	41,0	69,2	73,0

According to these HPLC-measurements increasing lipophilicity order of the studied molecules is expected to be **1a** < **2a** < **1e** < **2e**. Results correlate well with lipophilicity parameters (log P_{ow}) obtained from in-house documents (Santen Oy).

9.3.3. pH-lipophilicity profiles

The pH-lipophilicity profiles of two commercial isopropyl ester prodrugs (**1e** and **2e)** and their biologically active acid metabolites (**1a** and **2a**) were determined from series of isocratic measurements at different pH of the eluent. Examples of the obtained chromatograms are presented in Figure 24 (see Appendix 1). Retention times were converted into log k values and plotted as a function of pH. Ester forms were analyzed

by using acetonitrile/universal buffer 65:35 as mobile phase, whereas acid forms were analyzed by using acetonitrile/universal buffer 30:70. For acid metabolites (**1a** and **2a**) pH-lipophilicity profiles are presented in Figure 16.

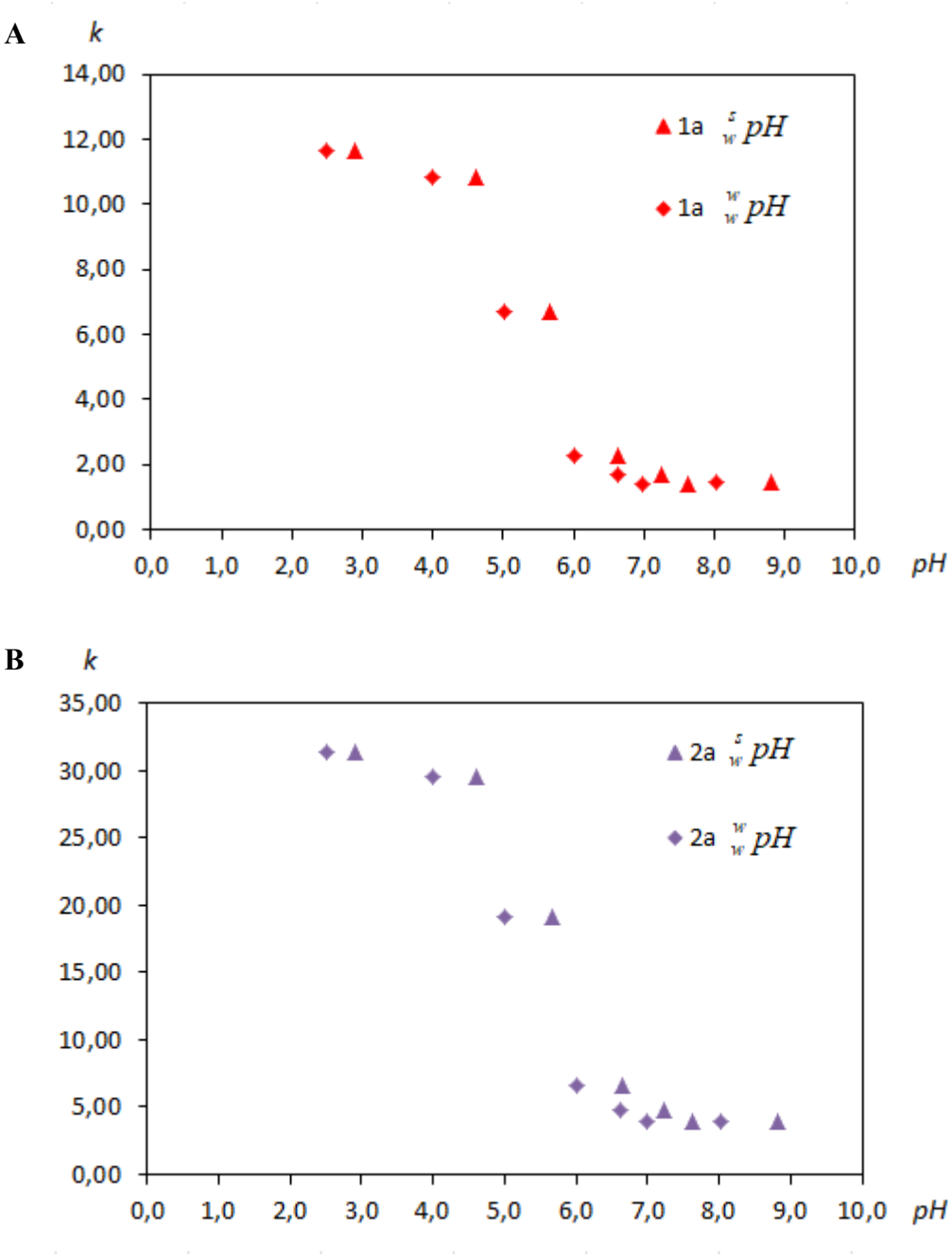

Figure 16. *Lipophilicity as log k of acid forms* ***1a*** *(A) and* ***2a*** *(B) versus (♦)* ${}^{w}_{w}pH$ *and (▲)* ${}^{s}_{w}pH$ *using acetonitrile/universal buffer 30:70 as mobile phase.*

For acidic components, increasing the pH of the aqueous portion of the mobile phase from 2,5 to 8 decreased the retention. At aqueous mobile-phase ${}^{w}_{w}pH$ values greater than 7, both acidic components were in their ionized form. At mobile phase ${}^{w}_{w}pH$ values of 4 and lower, the acids exhibited a high retention and lipophilicity, since they were in

neutral form. According to these measurements, ionization constant pK_a of both acids should be close 5 as the most dramatic decreased in retention occurred at the ${}^{w}_{w}pH$ region from 4 to 6. However, it's important to bear in mind that the addition of organic solvent to the eluent affects both the pH of the eluent and the ionization event of weak acids. Thus, the evaluation of pK_a values by RP-HPLC is not completely accurate, but gives rough estimates.

For isopropyl ester prodrugs (**1e** and **2e)** pH-lipophilicity profiles are presented in Figure 17. As was to be expected, alteration of eluent pH did not affect the retention of ester forms significantly since they do not contain any ionizable moieties.

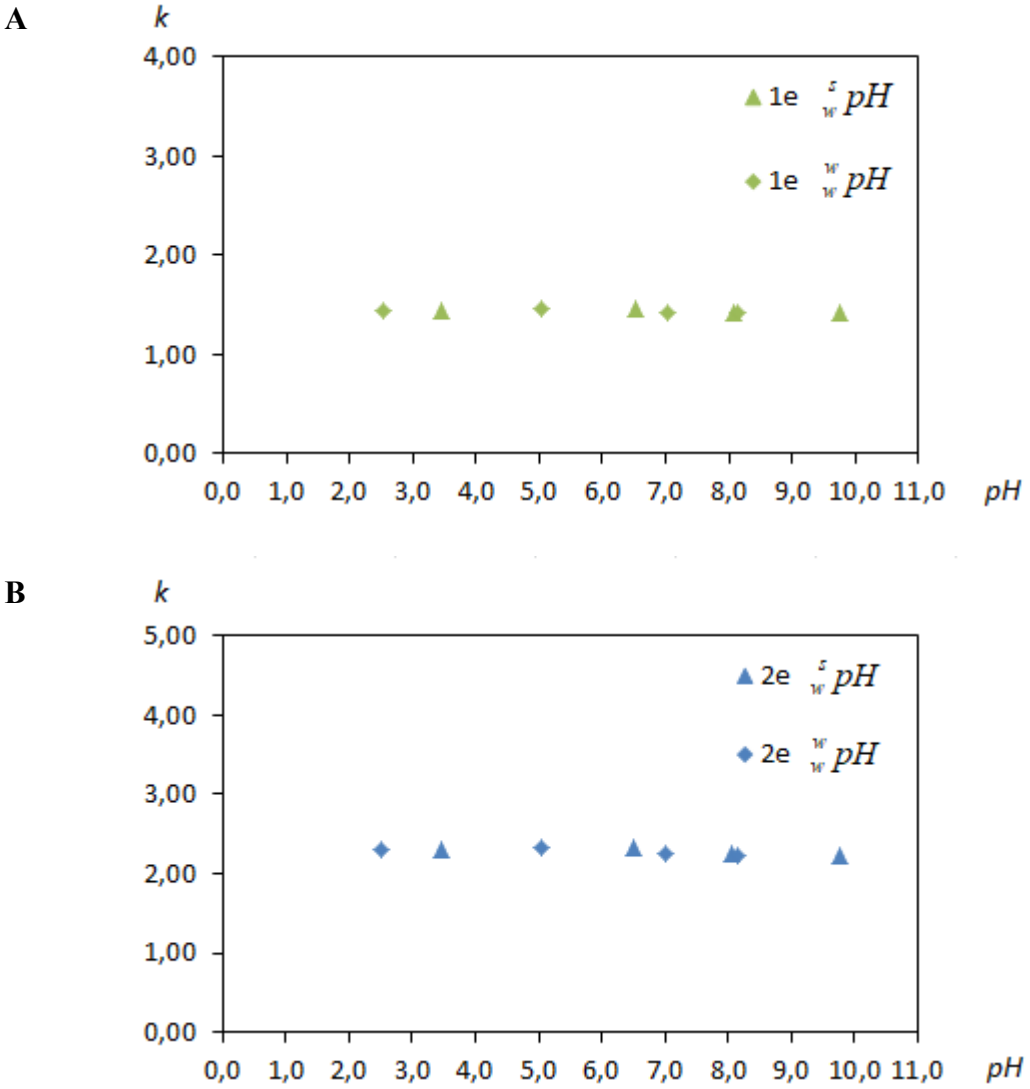

Figure 17. *Lipophilicity as log k of ester forms* ***1e*** *(A) and* ***2e*** *(B) versus (♦)* ${}^{w}_{w}pH$ *and (▲)* ${}^{s}_{w}pH$ *using acetonitrile/universal buffer 65:35 as mobile phase.*

9.3.4. Drug-additive interaction studies by RP-HPLC

Additive **X** is a primary amine that exists in physiological pH in cationic form while acids **1a** and **2a** exist in anionic form respectively. Therefore ion-pair formation between these oppositely charged moieties has been suggested. In this study ion-pairing theory was tested by adding increasing amount of additive **X** into mobile phase. If strong ion-pairing would happen, lipophilicity of the acid forms **1a** and **2a** should be improved which could be observed in RP-HPLC measurements as increased retention times**.**

Retention times of acid forms **1a** and **2a** were analyzed by using acetonitrile/sodium phosphate buffer 30:70 as a mobile phase. Desired amount of additive **X** was dissolved in buffer solution before adjusting the pH with 8,5 % phosphoric acid solution to 7,00 ± 0,02. Day-to-day variation in test conditions was taken into account by performing baseline measurements without additive **X** every day, five times all together. Retention times and retention factors are presented in Table 4.

Table 4. *Retention times t_r and retention factors k of acids **1a** and **2a** in different concentrations of additive **X** in mobile phase. Retention times without additive **X** were measured five times. Sample concentrations of **1a** and **2a** were 10,1 µg/ml and 12,0 µg/ml respectively.*

	t_r (min)		k	
c (additive X) in mobile phase	1a	2a	1a	2a
0 mM	5,75 ± 0,15	12,76 ± 0,50	2,46 ± 0,09	6,69 ± 0,30
0,5 mM	5,66	12,50	2,41	6,53
1 mM	5,67	12,57	2,42	6,57
10 mM	5,63	12,46	2,39	6,51
25 mM	5,87	13,30	2,54	7,01

Results do not indicate any apparent dependence on retention times and the amount of additive added to the eluent. Slight changes in retention times after the addition of additive **X** into eluent are most likely related to the day-to-day variation in measurement conditions. The range of the shortest to the longest retention time is the same with and without additive **X**. The results suggest that either the ion-pair formation does not occur or the HPLC method is not sensitive enough to indicate the formation of an ion pair. It is also possible that the concentration of additive **X** should have been significantly increased in the eluent so that the results would have been noticeable. The slight increase in retention times as the concentration of additive **X** was increased up to 25 mM may refer to this.

Since the HPLC method failed to confirm that additive **X** improves the lipophilicity of acid forms **1a** and **2a** by ion-pairing, it was decided to test whether the HPLC method could be used to determine if additive **X** directly affects the retention of the ester forms.

It was assumed that additive **X** might have some other specific interaction with ester forms which could decrease lipophilicity of prodrugs **1e** and **2e**.

Retention times of ester forms **1e** and **2e** were analyzed by using acetonitrile/sodium phosphate buffer 65:35 as mobile phase. Desired amount of additive **X** was dissolved in buffer solution before adjusting the pH with 8,5 % phosphoric acid solution to 7,45 ± 0,02. Measured retention times and retention factors are presented in Table 5.

Table 5. *Retention times t_r and retention factors k of prodrugs **1e** and **2e** in different concentrations of additive **X** in mobile phase. Sample concentrations of **1e** and **2e** were 12,6 µg/ml and 12,5 µg/ml respectively.*

	t_r (min)		k	
c (additive X) in mobile phase	1e	2e	1e	2e
0 mM	6,41	8,64	1,43	2,27
5 mM	6,42	8,66	1,43	2,28
25 mM	6,32	8,45	1,39	2,20

Also, in case of ester forms, the addition of additive X to the eluent did not significantly affect the retention times. With the highest additive concentration (25 mM) the retention times for both **1e** and **2e** were slightly shorter, but the difference may also be due to measurements made on different days. Nevertheless, minor shortening in retention times may indicate that the additive has some interaction with isopropyl ester prodrugs **1e** and **2e** which decreases their lipophilicity. The results could have been verified by using higher eluent concentrations.

According to these drug-additive studies by HPLC it cannot be confirmed that the additive **X** would affect the lipophilicity of isopropyl ester prodrugs **1e** and **2e** or their active acid forms **1a** and **2a**. It is possible that additive **X** does not interact with the drug molecules but affects the biological structure of the corneal surface and thus improves the permeation of these drugs.

10. DIRECT LOG P_{OW} MEASUREMENTS

10.1. Background and purpose of the study

Formulation development at Santen Oy has mainly relied on lipophilicity parameters obtained from literature or supplied by ingredient manufactures. However, the availability of octanol-water partition coefficients (log P_{ow}) is quite poor and parameters are often rough estimates. Thus, developing in-house laboratory models to determine octanol-water partition coefficients could improve the formulation development.

In this chapter, a modified shake-flask method is set up for determining direct log P_{ow} values of drugs at Santen Oy. In order to decrease required amount of drug molecules, the traditional shake-flask method described earlier in chapter 6.2 was modified into small-scale. The main target of the method development was to determine the time required in achieving partition equilibrium. Hence, partition experiments were performed by applying different shaking times varying from 15 minutes to 180 minutes. Duplicate determinations were made for each time points. Method was validated by determining octanol-water partition coefficient for isopropyl ester prodrug **2e** at physiological pH, since its log P_{ow} value was known to be 4,5 at pH 7. Additionally, lipophilicity (log P_{ow}) of prodrug **2e** with additive **X** was directly measured by this method in order to compare results obtained in previous chapter by RP-HPLC method.

10.2. Development and validation of modified shake-flask method

Before octanol-water partition experiments aqueous buffer phase and organic *n*-octanol phase were pre-saturated with each others. A sodium phosphate buffer solution (13 mM) was prepared by diluting 0,36 g sodium dihydrogenphosphate dodecahydrate in 100 ml of distilled water and adjusting the pH of the buffer to 6,96 by adding 0,22 ml of aqueous 8,5 % H_3PO_4. Equal amounts of above sodium phosphate buffer and *n*-octanol were mixed in eluent bottle and stirred vigorously by magnetic stirrer for 18 hours to mutually saturate them. Phases were separated in separatory funnel.

Schematic presentation of developed modified shake-flask method for determining direct log P_{ow} value of prodrug **2e** is presented in Figure 18. Accurately 1 ml of stock solution of prodrug **2e** in acetonitrile (61 µg/ml) was pipetted to 10 ml disposable centrifuge tube and the solvent was evaporated by nitrogen evaporator in 30 minutes at

22 °C, after which 2 ml of pre-saturated *n*-octanol was added to it. Solution was sonicated for 15 minutes to ensure the complete solvation of prodrug **2e** into *n*-octanol. An equal volume of pre-saturated aqueous buffer phase was then added and the mixture was shaken for a sample specific time (15, 30, 60, 120 or 180 minutes) by a multitube vortexer at room temperature. Shaking speed was adjusted as powerful as possible, however preventing solvents from reaching the cap of the centrifuge tube. In order to separate organic and aqueous phases properly, samples were centrifuged for 10 minutes at 2000 rpm.

Concentration of prodrug **2e** in both phases was determined by HPLC/MS/MS using already existing internal standard method (Santen Oy). Phase separation and sample preparations for HPLC-MS analysis were performed as follows: Phases were separated completely immediately after centrifugation to avoid the additional partitioning of drug molecules. 1 ml of *n*-octanol phase was pipetted to a 25 ml volumetric flask to wait for further treatments and the rest of the *n*-octanol phase was removed above the aqueous phase by a pasteur pipette. Then 1 ml of aqueous phase was mixed with 1 ml of internal standard acetonitrile solution (5 ng/ml) in a clean test tube. Since the drug concentration of *n*-octanol phase was assumed to be close to the initial concentration (~30 µg/ml), *n*-octanol phase was diluted 10 000 fold in acetonitrile. In order to remove traces of *n*-octanol, 1 ml of diluted sample was evaporated by nitrogen evaporator in 30 minutes at 30 °C. Remaining sample was diluted in 1 ml of internal standard acetonitrile solution (5 ng/ml) and 1 ml of Milli-Q water was added. Thus, the final internal standard concentration of all samples was 2,5 ng/ml and the diluents contained equal amounts of water and acetonitrile. Concentration of prodrug **2e** in both phases was determined by HPLC/MS/MS at Santen Oy.

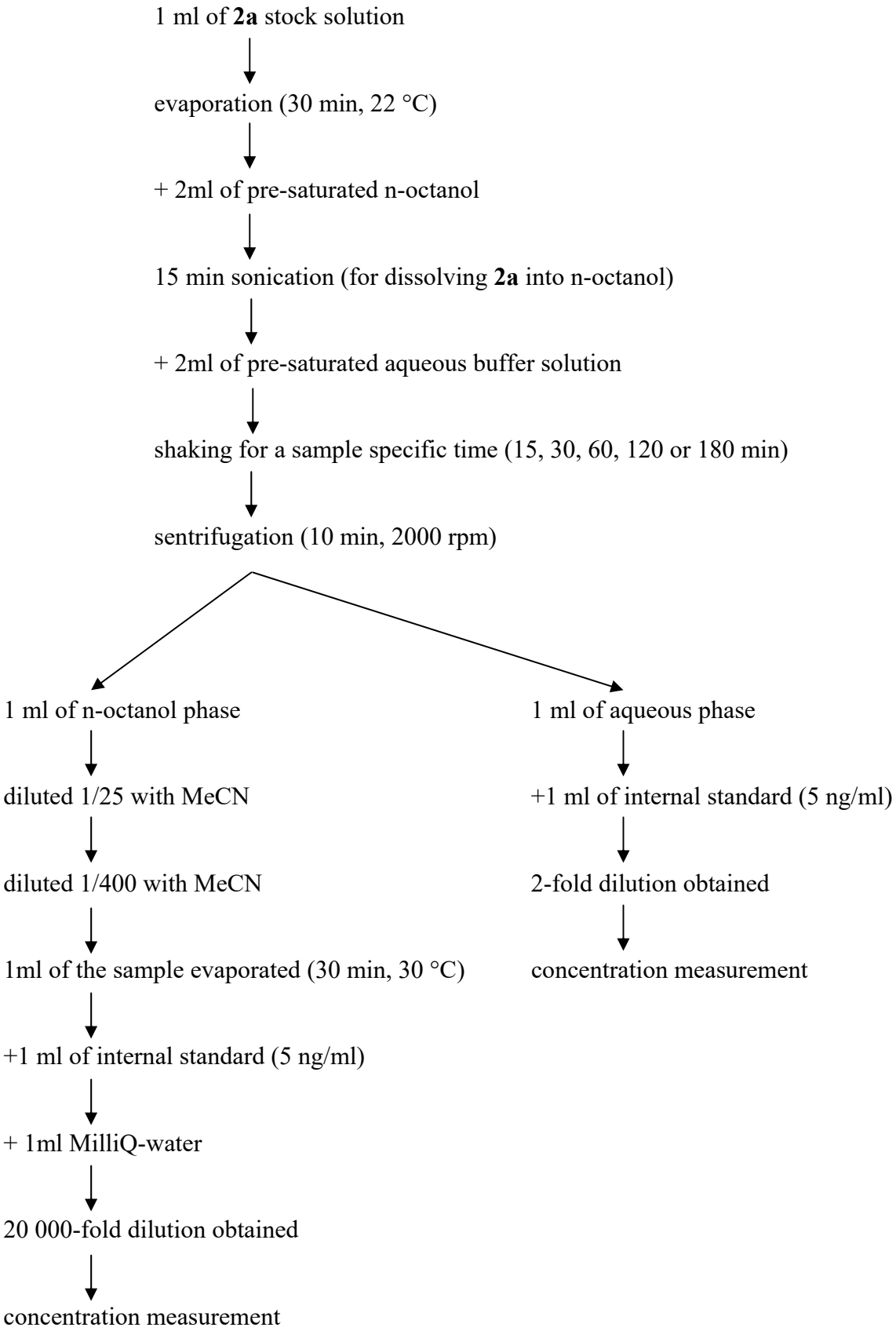

Figure 18. *Schematic presentation of developed modified shake-flask method for determining direct log P_{ow} values of drugs at Santen Oy.*

10.2.1. Results and discussion

Concentrations of prodrug **2e** in both water and octanol phases were determined by HPLC/MS/MS from diluted samples. Samples in octanol phase were diluted 20 000-fold and in water phase 2-fold before concentration measurements. Table 6 presents measured concentrations of diluted samples and calculated actual concentrations before dilutions. The last column of the table contains approximated sample concentrations in octanol phase which has been calculated as a difference between the initial sample concentration and the measured sample concentration in water phase.

Table 6. *The amount of prodrug* ***2a*** *measured from diluted samples and calculated before dilutions in water* c_w *and n-octanol* c_o *phases. Approximated* c_o *values were calculated as a difference between the initial sample concentration (27489,25 ng/ml) and the measured concentration of the water phase.*

	diluted samples		undiluted samples		approximated
$t_{shaking}$ [min]	c_w [ng/ml]	c_o [ng/ml]	c_w [ng/ml]	c_o [ng/ml]	c_o [ng/ml]
15	0,50695	0,00000	1,01	0,00	27488,24
15	0,45068	1,43751	0,90	28750,20	27488,35
30	0,43635	1,40288	0,87	28057,60	27488,38
30	0,45669	1,36431	0,91	27286,20	27488,34
60	0,42825	1,50994	0,86	30198,80	27488,39
60	0,79700	1,41264	1,59	28252,80	27487,66
120	0,40962	1,49566	0,82	29913,20	27488,43
120	0,43925	1,33584	0,88	26716,80	27488,37
180	0,40829	1,41042	0,82	28208,40	27488,43
180	0,45451	1,38397	0,91	27679,40	27488,34

Octanol-water partition coefficients log P_{ow} were calculated by equation 10 from actual undiluted sample concentrations. For each shaking time the average log P_{ow} was calculated. Two measurements were excludes from calculations due to sample contamination (line 6 in Tables 6 and 7) and instrumental error (line 1 in Tables 6 and 7). Additionally approximated log P_{ow} values were calculated by using approximated c_o values. Octanol-water partition coefficients log P_{ow} are presented in Table 7.

Table 7. *Calculated octanol-water partition coefficients log P_{ow} of prodrug* ***2e.***

$t_{shaking}$ [min]	log P_{ow}	mean	log P_{ow} *(appr.)*	mean
15	-		4,43	
15	4,50	**4,50**	4,48	**4,46**
30	4,51		4,50	
30	4,48	**4,49**	4,48	**4,49**
60	4,25		4,24	
60	4,55	**4,55**	4,51	**4,51**
120	4,56		4,53	
120	4,48	**4,52**	4,50	**4,51**
180	4,54		4,53	
180	4,48	**4,51**	4,48	**4,50**

Obtained log P_{ow} values correspond very well with the initial data according to which octanol-water partition coefficient for isopropyl ester prodrug **2e** is 4,5 at pH 7. At least for this substance, shaking time does not seem to have any effect on the result. Even 15 minutes of shaking might be accurate enough. However, with only one test substance it is not possible to draw general conclusions about the appropriate shaking time.

A particularly useful finding it that the approximated log P_{ow} values correspond well to the measured values. This enables to measure drug concentration only from the aqueous phase which would speed up the process and reduce the use of solvents because the dilutions of the octanol phase would not be needed. Additionally, this modified small-scale system consumes less solvent than the traditional shaking-flask method.

The main challenge of the method is the need of background information on the lipophilicity of the substance. Lipophilicity estimates help to designate which of the phases should be used for concentration measurements and how to design appropriate dilutions if needed.

10.3. Modelling drug-additive interactions by modified shake-flask method

Modified shake-flask method developed in previous chapter 10.2 was tested also for drug-additive interaction studies. Lipophilicity (log P_{ow}) of prodrug **2e** with increasing amount of additive **X** was determined by this method in order to compare results obtained in previous chapter by RP-HPLC method. Octanol-water partitioning experiments were performed otherwise like in chapter 10.2 except that the variating amount of additive X was added to the aqueous sodium phosphate buffer phases and the pH was adjusted to 6,98 ± 0,04. Triplicated determinations were made for each additive

concentration. Samples were shaken for 40 minutes. Drug concentrations were measured only in aqueous phase and concentrations in n-octanol phase were approximated by calculating the difference between the initial sample concentration (27489,25 ng/ml) and the measured drug concentrations in the water phase.

10.3.1. Results and discussion

The partition results (presented in Table 8) indicate that as the amount of additive **X** increases the lipophilicity (log P_{ow}) of the prodrug **2e** is also increased. Growth was almost linear, but there was also some deviation between the samples. Standard deviation between the samples is illustrated in Figure 19 by vertical line segments.

Table 8. *Measured drug concentrations in aqueous phase c_w and calculated drug concentrations in octanol phase c_o. The increasing amount of additive **X** is presented as a mass ration between additive **X** and prodrug **2e** in fist column of the table.*

m_X/m_{2e}	c_w [ng/ml]	c_o (appr.) [ng/ml]	P_{ow}	P_{ow} (avr.)	log P_{ow}	log P_{ow} (avr.)
0	1,0523	27488,1977	26123,01		4,42	
0	0,9638	27488,2862	28520,74		4,46	
0	0,8983	27488,3517	30601,10	28414,95	4,49	**4,45**
3,5	0,9709	27488,2791	28312,16		4,45	
3,5	0,9514	27488,2986	28893,08		4,46	
3,5	0,9194	27488,3306	29899,42	29034,89	4,48	**4,46**
17,5	0,8799	27488,3701	31240,33		4,49	
17,5	1,1224	27488,1276	24490,06		4,39	
17,5	0,8618	27488,3882	31897,22	29209,20	4,50	**4,46**
35	0,8770	27488,3730	31342,93		4,50	
35	0,9542	27488,2958	28807,08		4,46	
35	0,9312	27488,3188	29519,24	29889,75	4,47	**4,48**
52,5	0,9013	27488,3487	30499,91		4,48	
52,5	0,9374	27488,3126	29323,37		4,47	
52,5	0,9122	27488,3378	30135,43	29986,24	4,48	**4,48**
70	0,8080	27488,4420	34021,19		4,53	
70	0,8545	27488,3955	32167,48		4,51	
70	0,8333	27488,4167	32987,42	33058,70	4,52	**4,52**
350	0,6675	27488,5825	41183,87		4,61	
350	0,7784	27488,4716	35314,98		4,55	
350	0,6637	27488,5863	41415,94	39304,93	4,62	**4,59**

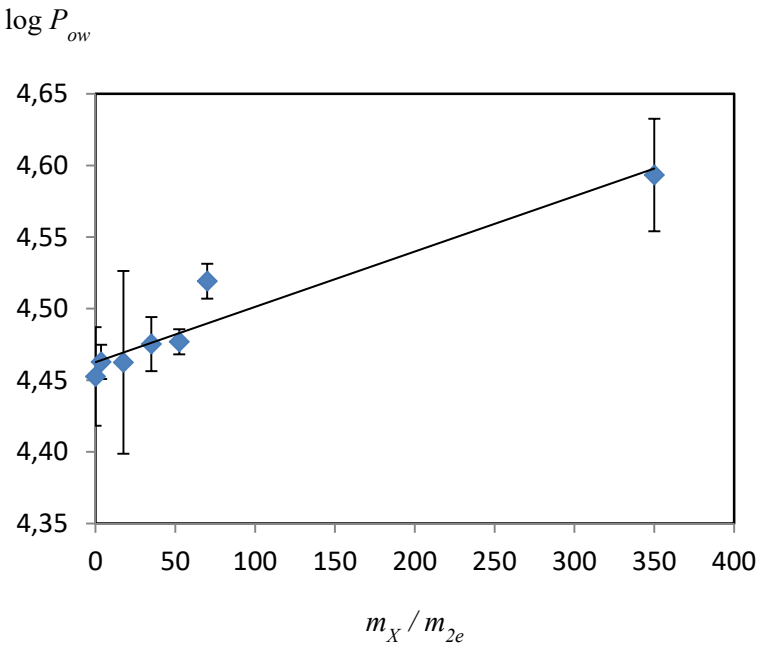

Figure 19. *Lipophilicity (log P_{ow}) of the prodrug **2e** as a function of increasing amount of additive **X** expressed as mass ration between additive **X** and prodrug **2e**.*

Obtained results do not correlate with earlier RP-HPLC measurement which indicated that additive **X** either does not affect at all or slightly decreases the lipophilicity of prodrug **2e**.

11. VALIDATION OF RP-HPLC METHOD FOR DRUG-ADDITIVE ION-PAIR FORMATION

11.1. Background

In this chapter, the usefulness of the C18-HPLC method for studying drug-additive interactions was put to the test. In chapter 9.3.4 assumed ion-pairing between acid form **1a** and additive **X** could not be verified as an increased retention time by RP-HPLC. At that time, it remained unclear whether the developed RP-HPLC method was not sensitive enough to detect ion-pairing or if ion-pair formation did not occur at all between acid form **1a** and additive **X**. Hence, it was decided to test the RP-HPLC method again, this time with substances that are already known to form ion pair.

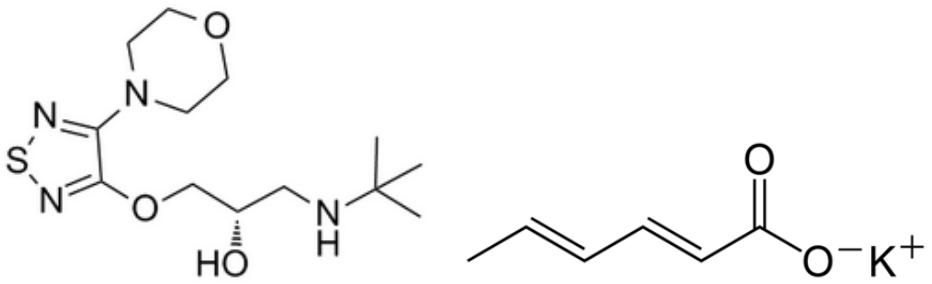

Figure 20. *Chemical strictures of timolol and potassium sorbate.*

Method was validated by using common anti-glaucoma drug timolol and sorbate additive, for which ion pairing had been previously verified by NMR (Higashiyama et al. 2007). Amino group in the tertiary butylaminopropanol moiety of timolol exists in physiological pH in cationic form while sorbate is anionic respectively. Sorbate is known to improve the bioavailability of timolol by increasing its ocular penetration due to the increased partitioning of timolol into the corneal epithelium. (Higashiyama et al. 2004) In this study ion-pairing between timolol and sorbate was studied by a RP-HPLC instrument by adding increasing amount of potassium sorbate into the eluent. Before ion pairing studies eluent composition was optimized for timolol. Additionally the pH-lipophilicity profile of timolol was measured.

11.2. Materials and methods

11.2.1. Buffer preparations

Sodium phosphate buffer solutions were prepared by dissolving required amount of disodium hydrogen phosphate dodecahydrate in Milli-Q water and adjusting the pH to 7,00 with 8,5 % phosphoric acid solution. Studies in which ion-pair formation between timolol and sorbate was investigated, potassium sorbate was dissolved in buffer solution and stirred for 10 minutes by magnetic stirrer before pH adjustment (pH 7,00 ± 0,01). Modified Britton–Robinson buffer (Britton & Robinson 1931) was used as universal buffer for the pH dependence studies. It was prepared by adding equal volumes of 1 M phosphoric acid, 1 M boric acid and 1 M acetic acid into Milli-Q water in order to bring the final concentration of each acid to 0,02 M. The desired pH was adjusted by addition of 1 M sodium hydroxide solution.

11.2.2. pH measurements

Buffer and HPLC eluent pHs were measured by Mettler Toledo MA 235 pH/Ion Analyzer. Electrode was calibrated against aqueous buffers using four-point calibration. In pH dependence studies the pH of the mobile phases were measured both before and after the addition of acetonitrile. ${}^{w}_{w}pH$ refers to the pH value of an aqueous portion of the mobile phase before the addition of acetonitrile (MeCN) and ${}^{s}_{w}pH$ refers to the pH value of a hydro-organic mobile phase measured after the addition of acetonitrile respectively.

11.2.3. HPLC instrumentation and settings

HPLC analyses were performed with Waters 2695 Alliance Separations Module equipped with a Waters 2996 Photodiode Array Detector (Waters Corporation, US) using Empower2 Chromatography Data Software for instrument control and data acquisition and processing. Phenomenex-Gemini C18 column (150 x 2,1 mm i.d.; 3 µm) was equipped with a Phenomenex security guard column. Eluent flow rate was set at 0,15 ml/min. Injection volume of the samples was 10 µl. Retention times t_r were measured at least triplicate and they were converted into retention factors k by applying Equation 12 in order to cancel out the effect of different flow rates. Void volume time t_o was determined by uracil. All analyses were performed in room temperature and the column was stabilized for at least 30 minutes before analyses. The detection wavelength was set at 294 nm.

Before HPLC-studies aqueous mobile phases were filtrated trough 0,45 µm HATF mixed cellulose ester membrane filter (Millipore). Hydro organic mobile phases were filtrated trough 0,45 µm HVLP Durapore membrane filter (Millipore). Additionally all

mobile phases were degassed by ultrasonicator before use. Timolol hemihydrate samples were dissolved in 1 % methanol solutions. Sample concentrations were 20 µmol/l.

11.3. Results and discussion

11.3.1. Eluent optimization and lipophilicity estimates for timolol hemihydrate

Six different mobile phase compositions were tested for timolol hemihydrate to achieve sufficient retention in reasonable run time for further studies. The most suitable eluent composition 25:75 MeCN/sodium phosphate buffer is highlighted in Table 9.

Table 9. *Retention times t_r, retention factors k and log k of timolol hemihydrate at different eluent compositions (t_o = 2,64 min). The most suitable eluent composition is highlighted.*

Eluent	t_r [min]	*k*	log *k*
45:65 MeCN / sodium phosphate buffer	3,28	0,24	-0,62
40:60 MeCN / sodium phosphate buffer	3,46	0,31	-0,51
30:70 MeCN / sodium phosphate buffer	4,28	0,62	-0,21
25:75 MeCN / sodium phosphate buffer	5,39	1,04	0,02
20:80 MeCN / sodium phosphate buffer	8,18	2,10	0,32
15:85 MeCN / sodium phosphate buffer	16,96	5,42	0,73

As a by-product of the eluent optimization several lipophilicity estimates were determined. Log *k* values were calculated from retention factors and plotted versus the volume fractions of the organic solvent acetonitrile in mobile phase *φ*. Linear approximation appeared to be quite poor (see Figure 21).

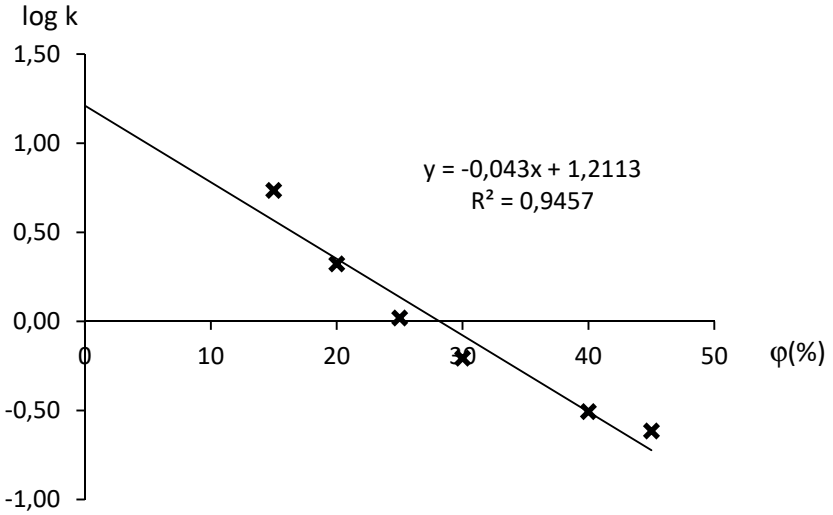

Figure 21. *Log k values of timolol hemihydrate as a function of volume fractions of organic solvent in mobile phase φ.*

Pure water log k_w and chromatographic hydrophobicity index φ_0 values extrapolated from the intersections of the axes are presented in Table 10. Obtained lipophilicity estimate values for timolol are not useful alone, but can be used in the future if the lipophilicity of timolol and other substances needs to be compared.

Table 10. *Extrapolated pure water log k_w and chromatographic hydrophobicity index φ_0 values.*

	log k_w	φ_0
Timolol hemihydrate	1,21	28,2

11.3.2. pH-lipophilicity profile of timolol hemihydrate

HPLC retention of timolol, expressed as log *k*, was monitored as a function of pH using acetonitrile/universal buffer 25:75 as a mobile phase. The analysis was performed as in the chapter 9.3.3. The examples of the measured chromatograms are presented in Figure 25 (see Appendix 1) and the obtained pH-lipophilicity profile is presented in Figure 22. Increasing the ${}^{w}_{w}pH$ of the aqueous portion of the mobile phase from 2,5 to 10,7 enhanced retention and lipophilicity of timolol. At mobile phase ${}^{w}_{w}pH$ values 11 and greater timolol exhibited high retention which indicates that timolol was in neutral form. However, as the ${}^{w}_{w}pH$ was adjusted to below 7, timolol eluted close to the void volume since it was in ionized form. According to these measurements, ionization constant pK_a of timolol hemihydrate should be close to 9 as the most dramatic decreased in retention occurred at the ${}^{w}_{w}pH$ region from 8 to 10. Considering the corresponding pK_a value 9,2 reported in literature (Sutinen et al. 2000), obtained estimate is pretty good.

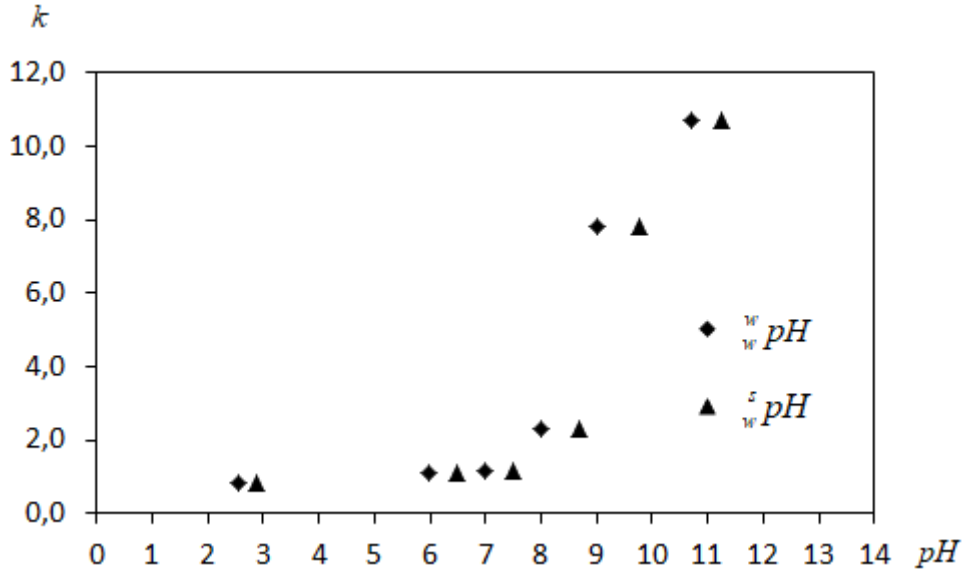

Figure 22. *Lipophilicity as log k of timolol hemihydrate versus (♦) ${}^{w}_{w}pH$ and (▲) ${}^{s}_{w}pH$ using acetonitrile/universal buffer 25:75 as mobile phase.*

11.3.3. HPLC analysis of ion-pair formation between timolol and sorbate

In this study ion-pairing between timolol and sorbate was studied with RP-HPLC by adding increasing amount of potassium sorbate into the eluent. Ion-pairing should improve the lipophilicity of timolol which supposedly could be observed in RP-HPLC measurements as increased retention times**.** Retention times of timolol were analyzed by using acetonitrile/sodium phosphate buffer 25:75 as mobile phase. Desired amount of potassium sorbate was dissolved in buffer solution before adjusting the pH with 8,5 % phosphoric acid solution to 7,00 ± 0,01. The examples of the obtained chromatograms are presented in Figure 26 (see Appendix 1). Measured retention times and retention factors are presented in Table 11.

Table 11. *Retention times* t_0*, retention factors k and log k of timolol in different concentrations of potassium sorbate in mobile phase (*t_0 *= 2,64 min). Sample concentration of timolol was 20 µmol/l.*

$c_{sorbate}$ (mM)	t_r (min)	*k*	log *k*
0,0	5,48	1,07	0,03
1,0	5,70	1,16	0,06
2,5	6,11	1,31	0,12
5,0	6,41	1,43	0,15
7,5	6,78	1,57	0,20
10,0	6,95	1,63	0,21
15,0	7,35	1,78	0,25

Results indicate clear dependence on retention times and the amount of sorbate added to the eluent (see Figure 23). Increasing sorbate concentration in mobile phase increases significantly the retention of timolol. The results suggest that RP-HPLC is able to indicate ion-pair formation.

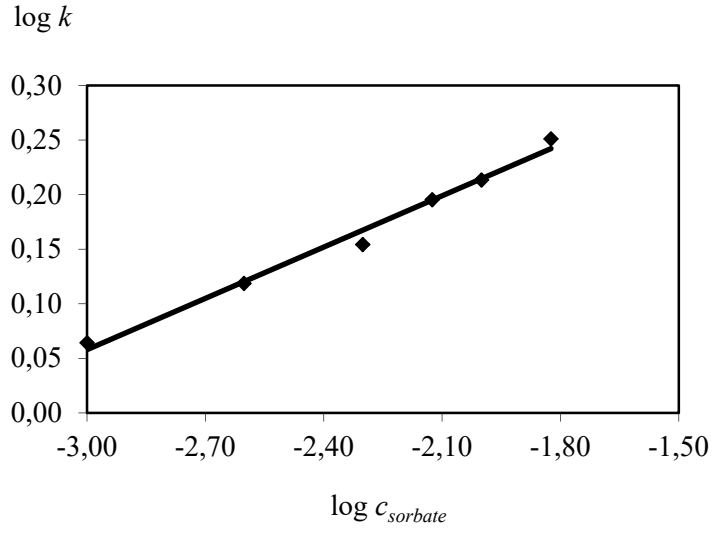

Figure 23. *Lipophilicity (log k) of timolol as a function of increasing amount of sorbate added to the mobile phase expressed as log* $c_{sorbate}$*.*

12. CONCLUSIONS

In this thesis various models for drug permeation studies were reviewed. The emphasis was on the determination of physicochemical parameters such as lipophilicity, expressed as logarithm of octanol-water partition constant (log P_{ow}), and ionization constant pK_a. These properties have a huge impact on membrane permeability and they can be used to predict drug absorption. Physicochemical analysis methods are attractive because they predict passive drug permeation reproducibly and efficiently. These methods also have a great high throughput potential and they require less resources and manpower compared to many other *in vitro* permeability prediction methods. On the other hand, they do not take into account physiological conditions or any specific interactions between drugs and membrane, unlike cell culture and tissue based models. However, physicochemical parameters can be used to select the most potential drug candidates for further animal studies and clinical trials on humans. They also give valuable information for formulation development.

Experimental part of this thesis was performed in the Quality Control and Analytical Development Laboratory of Santen Oy. The aim of the study was to develop in-house physicochemical laboratory models to support ophthalmic formulation development at Santen Oy. The main idea for experimental study was to find out if common RP-HPLC instruments and a simple shake-flask method could be utilized in modelling the lipophilicity of the ophthalmic drugs. The first goal was to find out how the effect of the formulation pH could be studied by HPLC. Another aim was to test whether the HPLC measurements or the shake-flask method could reveal drug-additive interaction.

Experimental work consisted of three parts. In the first part, lipophilicity of two commercial isopropyl ester prodrugs (**1e** and **2e**) and their biologically active metabolites (**1a** and **2a**) were studied by HPLC. Measurements consist of eluent optimization, lipophilicity estimation, pH-lipophilicity profile measurements and drug-additive interaction studies. As a by-product of the eluent optimization several lipophilicity estimates were determined. According to them the lipophilicity order of the studied molecules was expected to be **1a** < **2a** < **1e** < **2e**, which correlate well with reported lipophilicity parameters (log P_{ow}). Thus, it seems like a common C18-HPLC instrument can be utilized for comparing the lipophilicity of structurally similar drugs.

The pH-lipophilicity profiles of drugs **1a**, **2a**, **1e** and **2e** were determined from the series of isocratic measurements at different pH of the eluent. As was expected, changes in pH

did not affect the retention of the ester forms **1e** and **2e** significantly since they do not contain any ionizable moieties. For the acid forms **1a** and **2a** obtained pH-lipophilicity profiles suggested that the ionization constant pK_a for both acids is close to 5. Because the addition of organic solvent to the eluent affects both the pH of the eluent and the ionization event of weak acids, the evaluation of pK_a values by RP-HPLC is not completely accurate.

One of the main goals of this study was to find out of if some specific interactions, such as ion pairing between drug molecules and drug additives could be revealed with C18-HPLC-system. Pharmacokinetic studies in animals have suggested that cationic ophthalmic drug additive **X** increases the bioavailability of prodrug **1e.** Three different hypotheses had been made to explain the improved corneal permeability:

1) Additive **X** forms ion-pair with acid metabolite **1a** which increases the lipophilicity of acid **1a**.
2) Additive **X** might have some other specific interaction with ester prodrug **1e** which decreases lipophilicity of prodrug **1e**.
3) Additive **X** affects the biological structure of corneal surface.

Thus, it was studied if drug-additive interaction could be verified as changes in retention times by a RP-HPLC instrument when adding additive **X** into the eluent. Ion-pairing between acid forms (**1a, 2a)** and additive **X** could not be verified as an increased retention time by RP-HPLC. Additive **X** either did not affect at all or slightly increased the lipophilicity of acid forms. For the ester forms, the results were analogous. Additive **X** either did not affect or slightly decreased the lipophilicity of prodrugs **1e** and **2e**. Using higher eluent concentrations of additive **X** could have provided more information, but due to the high backpressure of the HPLC instrument, the concentration of the additive **X** could not be increased. According to these drug-additive studies, it could not be confirmed that the additive **X** would affect the lipophilicity of the studied drugs. It is possible that the additive **X** does not interact with the drug molecules but affects the biological structure of the corneal surface and thus improves the permeation of these drugs.

In the second part, a modified shake-flask method was set up for determining octanol-water partition constants (log P_{ow} values) of drugs at Santen Oy. Traditional shake-flask method was modified into small-scale in order to decrease solvent and drug consumption. Method was validated by isopropyl ester prodrug **2e** for which log P_{ow} value was known to be 4,5 at pH 7. Obtained log P_{ow} values 4,52 ± 0,04 and approximated log P_{ow} values 4,48 ± 0,05 correspond remarkably well with reference data even with only 15 minutes shaking time. Developed method seemed to be accurate and reliable enough to be used more extensively in Santen Oy.

To compare the results obtained previously by the RP-HPLC method, drug-additive interaction was tested also with the shake-flask method by measuring log P_{ow} of prodrug **2e** with additive **X**. Partition results indicated that as the mass ration between the additive **X** and the prodrug **2e** increased the log P_{ow} of the prodrug **2e** was also increased. Growth was almost linear. Obtained results did not correlate with the earlier RP-HPLC measurement which indicated that additive **X** either does not affect at all or slightly decreases the lipophilicity of the prodrug **2e**.

Since in the first part of the experimental work it remained unclear whether the developed RP-HPLC method was not sensitive enough to detect ion-pairing or if ion-pair formation did not occur at all between the acid form **1a** and the additive **X**, in third part a C18-HPLC method for studying drug-additive interactions was validated by using common anti-glaucoma drug timolol and sorbate additive, for which ion pairing had been previously verified. Increasing sorbate concentration in mobile phase increased significantly the retention of timolol. Enhanced lipophilicity of timolol was assumed to occur due to ion pairing with sorbate. Thus, the results suggest that RP-HPLC method is able to indicate ion-pair formation between drugs and pharmaceutical additives. This further makes hypothesis 1 unlikely for the previously studied **1a-X** molecule pairs, as ion-pairing should have been detected for them as well. Previously developed pH-lipophilicity profile measurements were performed also for timolol. Ionization constant was estimated to be 9 which correspond well with the pK_a value 9,2 reported in literature.

In this thesis, it was verified that the lipophilicity order between drugs can be easily studied by HPLC. Retention times themselves are good lipophilicity estimates, but by measuring the retention factors of the compounds at 3-5 different organic phase concentrations, extrapolated pure water log k_w and hydrophobicity index φ_0 values could be also converted into more comparable octanol-water partition coefficients. However, it would require calibrating the HPLC-system with compounds for which both hydrophobicity index φ_0 values and log P_{ow} values are known.

This project succeeded also in developing a rather simple HPLC method for studying the ionization of the drugs as a function of pH. Particularly useful findings were the Phenomenex-Gemini C18 column with great pH stability and the modified universal buffer solution which enabled measurements across a wide pH range. The method yielded good pK_a estimates, but the results must be treated with caution because the addition of organic solvent to the eluent affects both the pH of the eluent and the ionization event of the analytes. However, even rough pK_a estimates can be useful information for drug formulation development.

In addition, it was also demonstrated that the common C18-HPLC instrument can be used to reveal interactions between drugs and formulation additives. The ion-pair

formation between the drug and the additive can be detected as increased retention times when the amount of additive in mobile phase is increased.

Finally, a modified shake-flask method was set up for determining direct log P_{ow} values of drugs at Santen Oy. Method was developed by using already existing equipment and instruments. Obtained log P_{ow} values were very accurate and repeatable for the used reference sample, but the wider deployment of the method would require further testing with other reference substances. A particularly useful finding was that the approximated log P_{ow} values correspond well to measured values. At least with very lipophilic drugs this enables to measure drug concentration only from the aqueous phase which speeds up the process and reduce the use of solvents because dilutions of the octanol phase would not be needed. The main challenge of this method was the need of background information on the lipophilicity of the drug. This is however a very common problem in physicochemical analyses.

The main targets of the experimental work were achieved. It was verified that both common HPLC instruments and the modified shake-flask method can be utilized in modelling the lipophilicity of the ophthalmic drugs. Optimal formulation pH and drug-additive ion-pairing can be studied by RP-HPLC. Thus Quality Control and Analytical Development Laboratory could support ophthalmic formulation development at Santen Oy by using models developed in this thesis. However, well-established utilization of the models would still require the HPLC-method to be calibrated with reference substances so that the measured retention factors could be converted into more comparable octanol-water partition coefficients. In addition, it remained unclear in this study, why the ophthalmic drug solubilizing additive **X** increases the bioavailability of the prodrug **1e**. Defining the mechanism would require further experiments.

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APPENDIX 1: EXAMPLES OF HPLC CHROMATOGRAMS

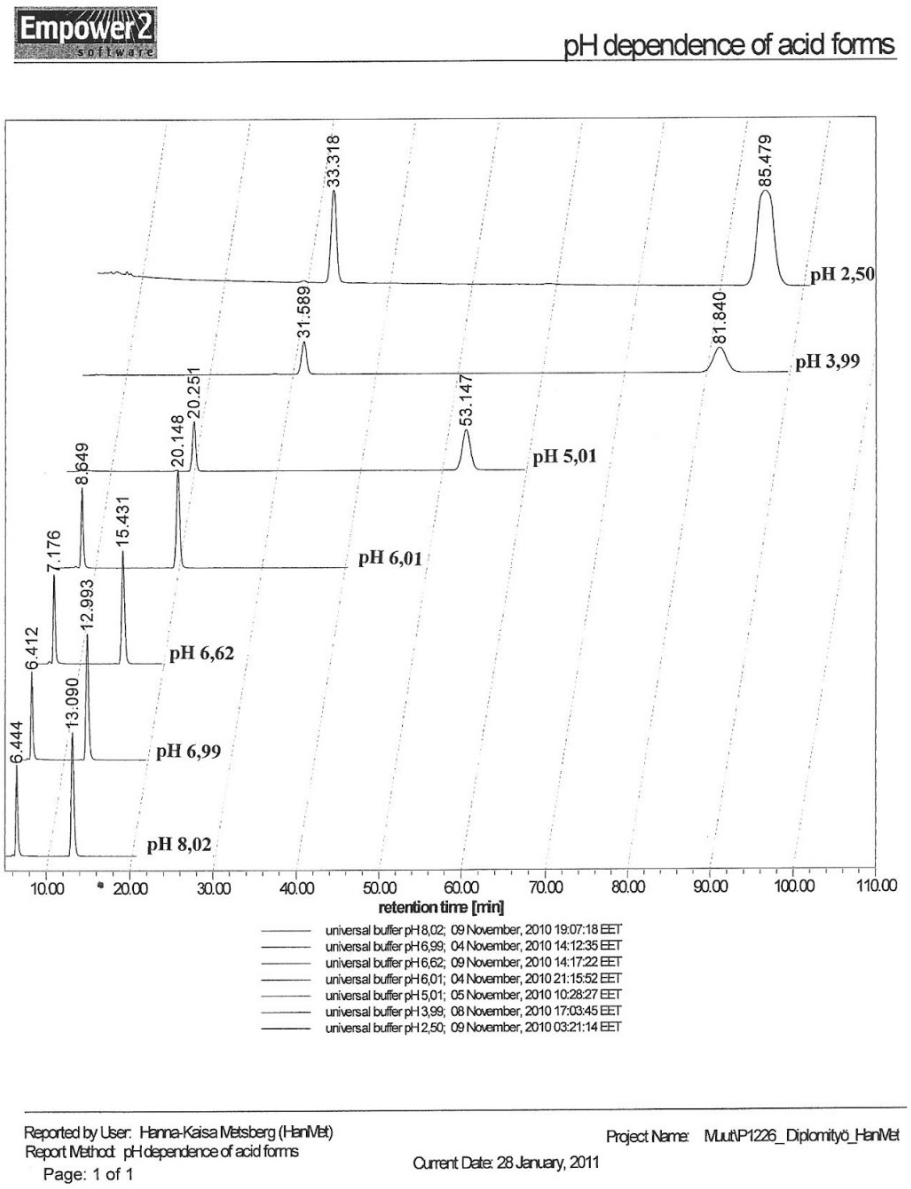

Figure 24. *Retention times of acid metabolites (**1a** and **2a**) determined from series of isocratic measurements at different pH of the eluent in chapter 9.3.3.*

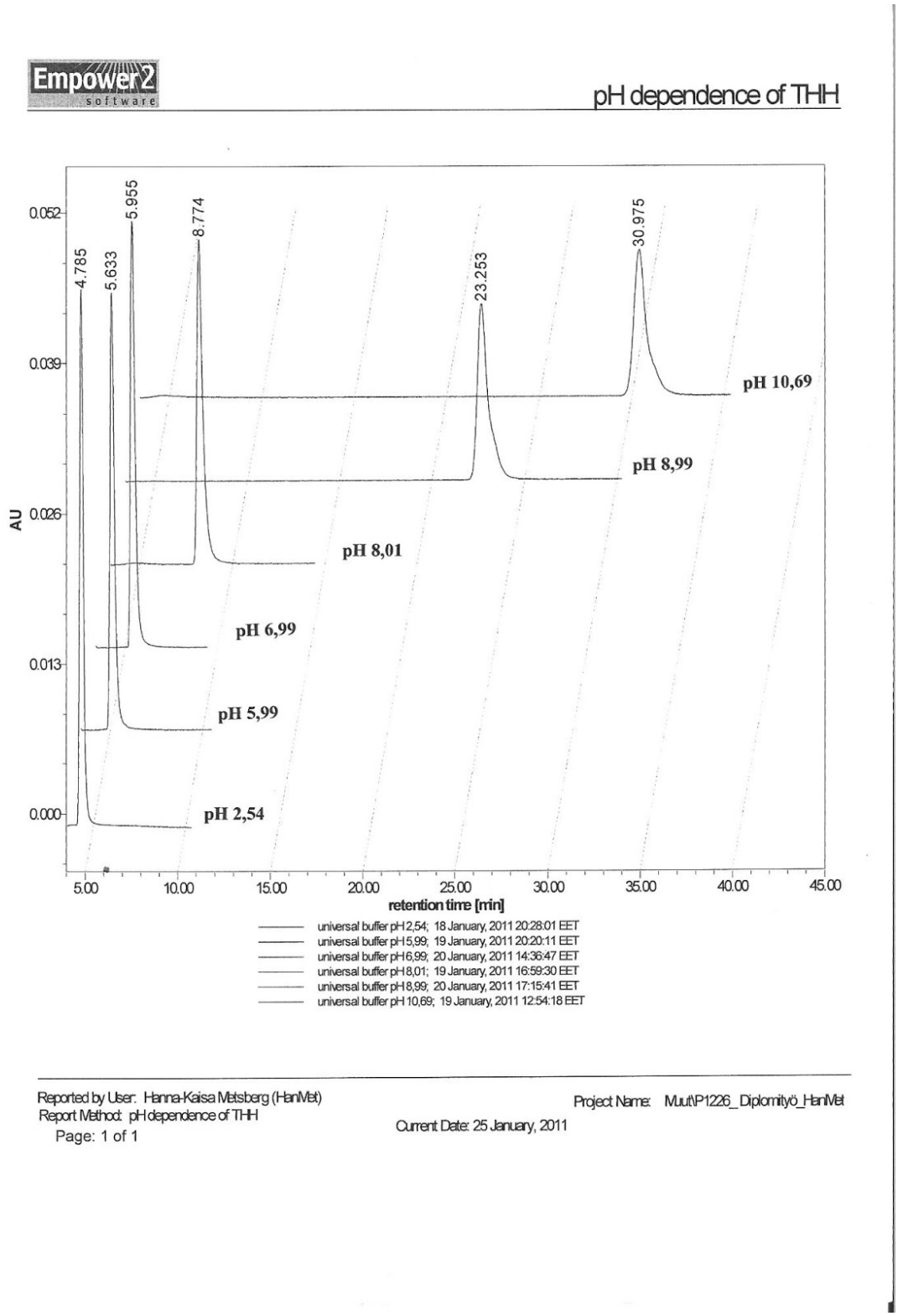

Figure 25. *Retention times of timolol hemihydrate (THH) determined from series of isocratic measurements at different pH of the eluent in chapter 11.3.2.*

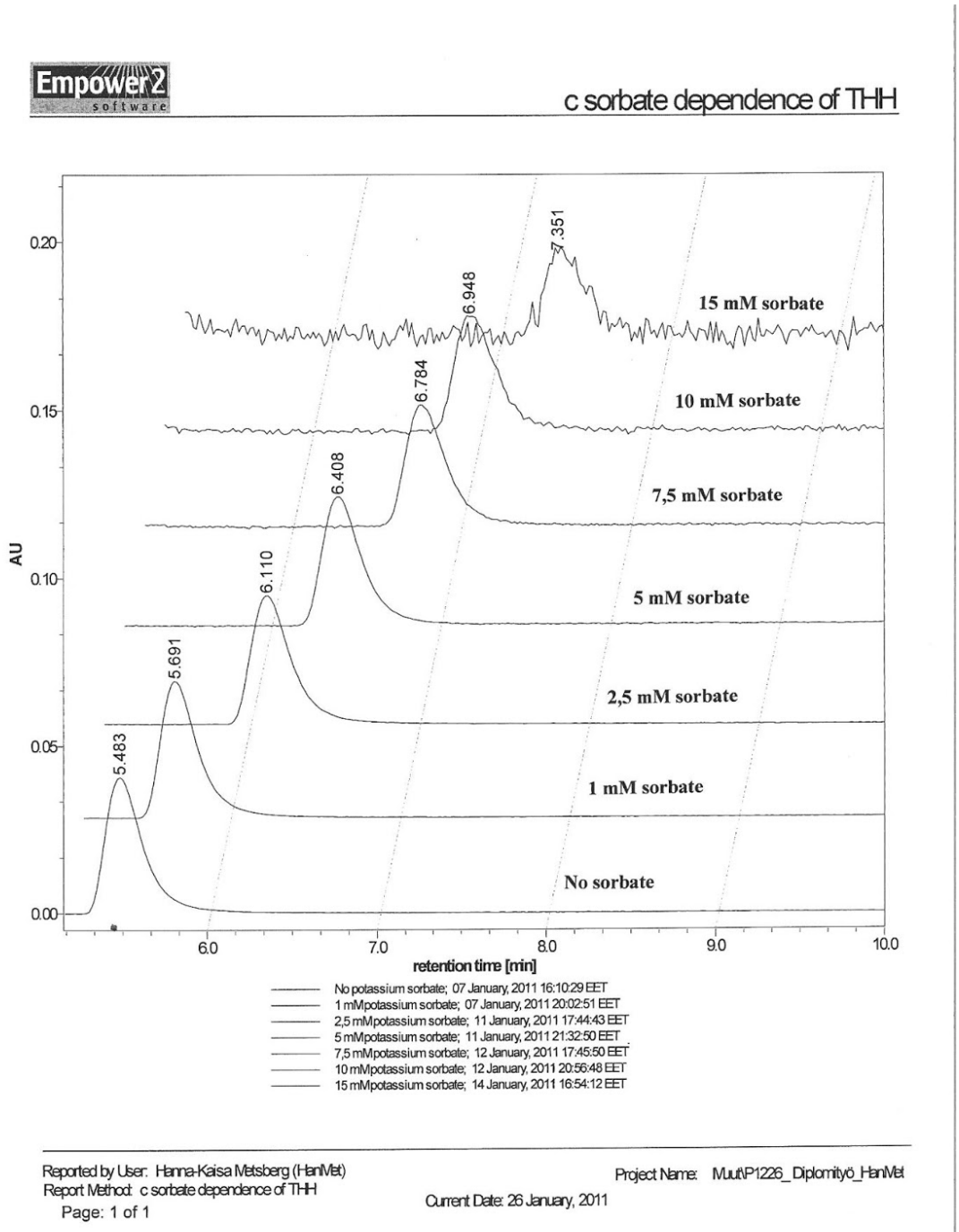

Figure 26. *Retention times of timolol hemihydrate (THH) as increasing amount of sorbate was added to the mobile phase in chapter 11.3.3.*